Health Canada / Santé Canada
Pest Management Regulatory Agency / Agence de réglementation de la lutte antiparasitaire

Date Received - Date de réception :

Office Use Only - à usage interne seulement

Notice of Objection to a Registration Decision under Subsection 35(1) of the *Pest Control Products Act*

Avis d'opposition à une décision d'homologation en vertu du paragraphe 35(1) de la *Loi sur les produits antiparasitaires*

1. Objector information - Information sur l'opposant

Name - Nom / Corporation - société / Organization - organisation: VALENT BIOSCIENCES			
Postal Address - Adresse postale: 870 TECHNOLOGY WAY			
City/Town - Ville: LIBERTYVILLE	Province/State - Province/État: IL	Country - Pays: USA	Postal Code/ZIP - Code postal/Zip: 60048
Phone - Téléphone: 847-968-4771	Fax - Télécopieur: 925-817-5938	E-mail - Adresse électronique: WARREN.SMITH@VALENT.COM	

2. Product information - Information sur le produit

Name of active ingredient to which the decision relates:
Nom de la matière active à laquelle la décision se rapporte : Bacillus thuringiensis

Name of end-use product to which the decision relates:
Nom de la préparation commerciale à laquelle la décision se rapporte : Multiple

3. Registration decision to which the objection relates - Décision d'homologation pour laquelle vous déposez un avis d'opposition

Decisions on application - Décision concernant la demande

- ☐ Granting registration - Homologation accordée
- ☐ Denying registration - Homologation rejetée
- ☐ Granting an amendment of a registration - Modification à l'homologation accordée
- ☐ Denying an amendment of a registration - Modification à l'homologation rejetée

Decisions on re-evaluation or special review - Décision concernant la réévaluation ou l'examen spécial

- ☐ Confirming registration - Homologation confirmée
- ☐ Cancelling registration - Homologation annulée
- ☒ Amending registration - Modification à une homologation

4. Date the decision statement was made public: Date de la publication de l'énoncé de décision : 6 May 2008

5. Area of scientific evaluation to which the objection relates - Volet de l'évaluation scientifique touché par l'avis d'opposition

- ☒ Health risk assessment (toxicology, food residue, occupational exposure) - Évaluation des risques pour la santé (toxicologie, résidus dans les aliments, exposition professionnelle)
- ☒ Environmental risk assessment (environmental fate, environmental toxicology) - Évaluation des risques pour l'environnement (devenir dans l'environnement, écotoxicologie)
- ☐ Value and efficacy assessments (crop tolerance, value) - Évaluation de la valeur et de l'efficacité (tolérance des cultures, valeur)

6. Scientific basis for the objection / Fondement scientifique de l'opposition

Attachment included / Pièce jointe incluse : ☒ Yes ☐ No / ☐ Oui ☐ Non

Warren L Smith	WARREN L. SMITH
7. Signature of objector or representative - Signature de l'opposant ou de son représentant	Printed Name - Nom en lettres moulées

Objectors who submit confidential information (i.e. confidential business information, confidential test data) are responsible for identifying this information which is part of their submission

Information required to process the notice of objection may include some personal information as defined in the *Privacy Act*. In accordance with that Act, such personal information may be made public as authorized by the *Pest Control Products Act* and its regulations. Under the *Privacy Act*, individuals have the right to look at their personal information. For more information on how PMRA manages personal information, contact the PMRA Information Services at 1-800-267-6315 within Canada and 1-613-736-3799 outside of Canada or via e-mail at pmra_infoserv@hc-sc.gc.ca

Les opposants qui soumettent des renseignements confidentiels (c.-à-d. des renseignements commerciaux confidentiels, des données d'essai confidentielles) sont responsables de les désigner comme tels dans leur envoi

L'information requise pour traiter cet avis d'opposition peut comprendre certains renseignements personnels tels que définis dans la *Loi sur la protection des renseignements personnels*. Conformément à cette Loi, ces renseignements peuvent être rendus publics, ce qui est permis par la *Loi sur les produits antiparasitaires* et son Règlement. En vertu de la *Loi sur la protection des renseignements personnels*, tous les individus ont le droit de consulter leurs renseignements personnels. On peut obtenir des précisions sur la gestion des renseignements personnels auprès de l'Agence de réglementation de la lutte antiparasitaire (ARLA) en communiquant avec le Service de renseignements au 1-800-267-6315 au Canada, ou au 1-613-736-3799 de l'extérieur du Canada, ou par courrier électronique à pmra_infoserv@hc-sc.gc.ca

Canada

Valent BioSciences Corporation (VBC) Notice of Objection to the PMRA Decision on Label Requirements for Bacillus thuringiensis

Application to Aquatic Habitats

Valent BioSciences Corp. is concerned about the need of label language specific to Btk under **DIRECTIONS FOR USE** - "Do not apply directly to aquatic habitats ..... "

This language as written will preclude the primary intended uses for some of the Btk products. Standard label language can prevent a product from being used for an approved use because it makes practical application of the product impossible. Bts do not pose actual aquatic concerns (please see some of the scientific indications of this below). Hence additional label language needs to be developed which clearly indicates that forestry uses of Bt, do not present a direct application to aquatic habitats.

Labels already clearly indicate the applications permitted. There are no aquatic uses on the labels for Btk. The concern comes on possible precautionary statement interpretation/liability when doing aerial Btk applications on, for example, large forestry tracks that may contain streams, prairie potholes, creeks, etc. While the application is not 'directed' at aquatic habitats overspray cannot be strictly controlled as aquatic habitats intermingled in forests are overflown.

Btk sprays have been undertaken for years in Canada. Projects undertaken by Environment Canada continue to show the innocuousness of Bt to the environment. For example the study: **PROJECT, Ecotoxicology of Biotechnology Products: The case of *Bacillus thuringiensis*** (www qc.ec.gc.ca/csl/pro/pro032fg_e.html)

Gives the following conclusion:

"Main Results to Date

Results have shown that although Bt crystals appear rather stable, the toxin is found in very low concentrations in the aquatic environment and degrades more quickly in water than in soil. Work is continuing in order to determine the presence and persistence of the gene and of the Bt corn toxin (Cry1Ab) in the aquatic environment."

During nearly 45 years of registration and about 30 years of wide scale aerial and terrestrial application around the world, Btk applications have not resulted in any significant aquatic effects when applied in accordance with the label. The proposed wording is not supported by fact. The science clearly shows the little risk Bts poses to aquatic habitats in all their complexity.

The paper: Functional Effects of the Bacterial Insecticide *Bacillus thuringiensis* var. *kurstaki* on Aquatic Microbial Communities. David P. Kreutzweiser[a], J. Lawrence Gringorten[a], David R. Thomas[a] and Jason T. Butcher

[a] Canadian Forest Service, 1219 Queen St. East, Sault Ste. Marie, Ontario, Canada, P6A 5M7

Ecotoxicology and Environmental Safety Volume 33, Issue 3, April 1996, Pages 271-280

Indicates: "These results from laboratory and controlled field experiments indicate that contamination of watercourses with Btk is unlikely to result in significant adverse effects on microbial community function in terms of detrital decomposition."

In addition, an up-to-date book by two New Zealand scientists covers the potential environmental impacts of Bt in great detail.

- Glare, T.R. and O'Callaghan, M. 2000. *Bacillus thuringiensis*; Biology, Ecology and Safety. John Wiley and Sons, Chichester, UK. 350 pp.

The World Health Organisation have also recently reviewed the environmental impacts of Bt (Anonymous 1999. Microbial Pest Control Agent *Bacillus thuringiensis*. Environmental Health Criteria 217. World Health Organisation, Geneva).

Both publications offer no significant effects on aquatic systems by Bts.

Water quality should not be directly affected by Btk as it is not likely to affect most aquatic organisms. Some North American laboratory studies have shown decreases in detritus decomposition rates at high doses of Btk. However, these effects are unlikely in the environment because of the lower doses of Btk used and the purification processes in natural systems (USDA 1995).

VBC Proposal: As indicated in the Bt Re-evaluation Document, there are no aquatic impacts associated with the use of Bt. This proposed label language should be modified/present an addendum to prevent the liability questions raised by the language in its current state. The control of pests that are present in vegetation over waters should not be considered a direct application of pesticide to water. Standard label language which is appropriate and feasible for agricultural uses, is not workable for forestry applications. Labels should be reflective of actual concerns and safety precautions that are in fact called for.

Precautionary Statements:

Valent BioSciences Corp. believes precautionary wording should be specific to the product being labeled. Thus we agree with the indication that "CAUTION EYE IRRITANT" may be waived if data is submitted that indicates that eye irritation would not be an issue with a particular Bt product.

Additionally, the Re-evaluation Decision Document would require that Bt labels must contain precautionary statements indicating that the product is a POTENTIAL SENSITIZER. VBC believes that data should also be considered for determining language on sensitization. While bacterial products, due to their protein content may have the potential to be sensitizers, if data is sufficient to indicate that sensitization would not be an issue for a specific strain and/or formulation, then the requirement for a sensitization statement should also have the possibility to be waived.

In its PACR for Bts, PMRA has evaluated a large number of publications and numerous surveillance reports of large scale human exposure. None of the epidemiological studies identified sensitization from, at least, specific Btk formulations. The potential for sensitization should be able to be waived when there is a large volume of work which would indicate that a specific product does not in fact cause sensitization.

A science based risk evaluation should take into consideration all studies available, for example, where sensitization studies with standard protocols indicate a lack of sensitization there should be no requirement for cautionary statements indicating the product is a potential sensitizer.

Additional scientific evidence as to the different potential of different microbial species to elicit sensitization is attached as Appendix I.

VBC Proposal:
Label language should be left open to being modified by specific data.

Personal Protective Equipment:

The precautionary statements include a statement to 'Avoid breathing dust/spray mist' and a mandatory requirement for a NIOSH-approved '–95 'respirator face-mask when handling, applying, or performing cleanups associated with the product. This requirement will provoke a negative response amongst applicators and the public alike.

Given that, as indicated in the PACR document:

"The risk from occupational exposure, however, was determined to be low given the lack of mammalian toxicity." and that "Occupational and non-occupational exposure in Canada is expected to be lower in than in the United States, as the Canadian use rates are generally lower."

And that

"This decision was based on the sum total of all toxicology data submitted to the USEPA along with the lack of any report of significant human health hazards of the various B. thuringiensis strains."

Therefore, the proposed label language is excessively negative and not supported by the available data. The precautionary language and mitigation required is inconsistent with the potential risk due to exposure. The requirement to have workers exercising this level of precaution sends a message to workers and the public that the material being applied is relatively dangerous since similar precaution is not required of many chemical alternatives. There should be no requirement for a dust mask when friability and other applicable studies indicate negligible potential for exposure to dust and inhalation studies indicate minimal toxicological concern from exposure. While harmonization of Canadian labels with the U. S. for similar products is a worthwhile objective in principle, the policy of requiring dust masks for microbials when testing indicates no apparent risk and no guidelines exist for studies to refute the perceived risk is bad policy on either side of the border.

In most forestry and agricultural application scenarios, liquid Bt formulations are handled via a closed-loop pesticide mixing and loading system; mixers and loaders may not be exposed to the product; consequently the need for a NIOSH –95 filter is not required.

The new label language requiring the use of PPE for all handlers of Bti formulations is unnecessary and poses undue requirements upon registrants and end-users.

It has been stated that based on all available data, current labeled applications pose no risk to applicators, handlers or bystanders; therefore the use of a NIOSH-95 filter is an unwarranted restriction not supported by fact.

Not only does this drive up the cost of public health programs, (a significant concern for many large scale municipal programs) it absolutely provides the wrong message to residents of the treated areas. A dust mask for hygiene purposes is adequate protection for Bti handlers. Residents will not easily accept the safety of Bti based products when they are confronted with NIOSH-95 equipped applicators working in their neighborhood.

VBC Proposal: PMRA should propose label language that is supported by data. PMRA recognizes that there are already several strain sources and therefore PMRA should also recognize the variability within these sources for numerous factors, including unique characteristics and usage patterns. The cautionary notes re Sensitization

and the requirement for '–95' NIOSH respirators should be left open to being ameliorated based on scientific data.

Label Language:

End-use products:

VBC would question the requirement to have all end-use labels include a "Do not apply by any type of irrigation system." For certain agricultural Btk uses, chemigation may be an effective way of applying the product. No scientific rational for the statement has been proposed by PMRA.

VBC Proposal: The possibility for this application method should be open to a risk assessment.

Response to Potential Sensitizer precautionary statement for ***Bacillus thuringiensis*** **subsp. *kurstaki* strain ABTS-351**

Sensitisation/allergenicity observations

Bernstein et al. (1999) observed farm workers (vegetable harvesters) before and after exposure to *Bacillus thuringiensis* subsp. *kurstaki* (strain SA-11). There was no evidence of occupationally-related respiratory syndromes. Positive skin test responses to spore extracts of *B. thuringiensis* subsp. *kurstaki* were observed, and specific IgE and IgG antibodies were present. However, following repeat exposure in 579 Danish greenhouse workers exposed to DiPel® (strain ABTS-351), there was no evidence of sensitisation based on a measurement of total IgE (Larsen & Bælum, 2002).

Doekes et al. (2004), studying more than 300 greenhouse workers, reported that exposure to *B. thuringiensis* biopesticides (Bactimos® and Vectobac®; *B. thuringiensis* subsp. *israelensis*) confers a risk of IgE-mediated sensitization. No increase in respiratory health symptoms was observed.

Laferrière et al. (1987, as cited in WHO, 1999) demonstrated antibody titres against vegetative cells of *B. thuringiensis* in workers exposed to *B. thuringiensis* subsp. *kurstaki*. Little or no formation of antibodies against spores or crystals was detected. No adverse health effects were reported.

Two incidents of possible allergic reaction to *B. thuringiensis* have been reported to US EPA (McClintock et al., 1995). However, *B. thuringiensis* was not considered to be the causative agent in any of the cases.

Comment from Registrant:

All papers cited indicate no adverse health effects.

What is the Significance of Elevated IgE levels in People Exposed to Btk?

Allergic sensitization is a multi-step process. Additionally, the absence of symptoms in exposed individuals, who present measurable allerginicity parameters that may be indicative of sensitization, needs to be explained. No single parameter is currently predictive of allergic potential. It may be that asymptomatic responses represent a normal distribution of responsiveness in the general population without clinical consequences.

Microbial caused sensitization:

Sensitization potential with microbial products appears to be better documented with certain microbes than for others. Gram-negative bacteria with their bacterial liposaccharide (LPS) endotoxin have been proposed as a major candidate for pulmonary inflammation reaction due to their presence in organic dusts. Rylander (2002) reviewed endotoxins in the environment and a possible relationship among exposed persons. Rylander concludes there is a relationship between exposure and disease, yet at certain exposure levels and /or at certain periods of life, exposure may be beneficial and may even reduce the risk for disease. A review of the literature also indicates there is "still a need to investigate other potentially active agents in environments with endotoxin exposure."

Even within the well documented gram-negative endotoxin story, additional studies are now questioning the difference between purified LPS and the true effects of environmentally exposed bacterial systems. There appears to be a differentiation in the Th1 or Th2 cascade. Th1 immune deviation has been shown to decrease airway hyper-reactivity, AHR, and therefore has been suggested that LPS induced immune deviation toward a Th1 response inhibits the Th2 response that leads to asthma (Zuany-Amorim et al. (2002); Erb et al. 1998; Herz et al. 1998). Growing evidence is indicating that bacterial exposure could drive the atopic response into a TH1 direction and away from allergicity (Douwes and Pearce, 2002).

Tulić et al. (2000) looked at the potential role of exposure to bacterial lipopolysaccharides on the development of sensitization to allergen and the response to allergen challenge *in vivo*. They found that exposure to LPS after allergen challenge in sensitized animals abolished the hyper-responsiveness and modified the inflamatory cell influx of late-phase response to allergens.

George et al. (2006) reared C3HeB/FeJ mice (a strain particularly sensitive to endotoxin) in corn dust considered to have a high endotoxin content and microbial product or in low-endotoxin environment. The influence of the corn dust was accessed by BAL cell analysis and immuno-staining of lung tissue. The corn dust was not associated with an inflammatory response in pulmonary alveoli at any time point. There was a high concentration of lung eosinophils early in life which were proposed to possibly be associated with the TH2 bias (or reduced Th1 presence) previously described in neonatal mice and human infants. The conclusion of the study showed that exposure to a nonhygienic environment did not induce significant airway neutrophilia, yet it altered the population of immunologically active cells in the lung and reduced subsequent inflammation.

Gram-positive bacteria have little documented allerginicity potential, especially with any non-pathogenic species. Rather, several species have been proposed to reduce immune responses to antigens. For example, United States Patent 20070190076 (2006) by Institut Pasteur and Institut National de la Santé et de la Recherche Médicale is defined as a bacterial preparation that contains killed Gram positive bacteria, obtainable by a process which does not denature the structure of the molecules from the bacteria cells, and it is able to induce, *in vivo*, a modulation of the immune response against an antigen.

Lactobacillus casei, a nonpathogenic gram-positive bacterium widely used in dairy products has been shown to enhance the cellular immunity of the host. To examine the inhibitory effect of *L. casei* on IgE production, Shida et al. (1998) used splenocytes obtained from ovalbumin (OVA)-primed BALB/c mice that were restimulated *in vitro* with the same antigen in the presence of heat-killed *L. casei*. *L. casei* induced IFN-γ, but inhibited IL-4 and IL-5 secretion, and markedly suppressed total and antigen-specific IgE secretion by OVA-stimulated splenocytes. The Th cell development assay showed the ability of *L. casei* to induce Th1 development preferentially.

Ciprandi et al. (2005) used *Bacillus claussi* spores administered in oral suspension to study potential effects on nasal symptoms in children presenting nasal allerginicity symptoms. *B. clausii* restored physiological Th1 polarization and reduced nasal aesoniphils. B. claussi has also been shown by Ciprandi et al. (2004)to exert modulary activity in allergic children with recurrent respiratory infections.

Von Hertzen and Haahtela (2006) present an overview indicating that settings associated with exposure to microbes in soil and vegetation might be beneficial, even necessary, for the normal maturation of the immune system. High-level exposures to microorganisms in soil have been associated with reduced risk for asthma and atopy.

Are IgE levels/skin reactivity always indicative of clinical allerginicity?

Human response to microbial antigens may induce IgE or IgG antibodies that connote prior exposure, but not necessarily a symptomatic state. Vojdani et al. (2003) indicated: "Furthermore, presentation of clinical symptomatologies did not correlate with the levels of mold antibodies in all patients. Therefore, it seems that antibody levels do not correlate with disease severity, but are indicative of exposure. Therefore, in some individuals, detected mold IgG, IgM, and IgA antibodies may be protective--but not pathogenic."

Two Italian studies with populations selected according to standarized procedures were investigated and followed over time (Baldacci et al., 1997). The populations lived in two areas, namely a rural area in northern Italy and an urban one in Central Italy. The prevalence of respiratory symptoms was higher in the urban area compared to the rural area. On the other hand, no difference between the rural and urban areas was evident when comparing skin reactivity prevalence as a marker of atopy.

A growing body of knowledge indicates the promotion of clinical tolerance to allergens by certain microbial exposures. For example, the July 16, 2007 issue of Thorax contains two articles which add to the observations of an inverse link between mycobacterial exposure and atopic disorder. The relationship between atopy, asthma and mucobacteria are reviewed by JM Hopkins in the same issue.

Georges et. al (2006) showed that both early life and later life sub-chronic exposure to corn dust (high endotoxin-containing) reduced atopic airway inflammation in mice in

response to a known sensitizer (ovalbumin). Yet this exposure did not alter the Ig-E levels in serum in response to the sensitizer.

There is a large range in normal IgE levels (Merrett, 1997) indicated that circulating levels <20 kUL^{-1} render a diagnosis of atopy unlikely, while concentrations > 180 kUL^{-1} indicate a likely diagnosis of atopy. Jansen et al. (1998) showed that analyzing for symptomatic and asymptomatic subjects indicated a higher risk for bronchial hyper-responsiveness correlated with a positive skin test only in symptomatic subjects, independent of high serum total IgE levels.

IgE may actually help defend against certain infections and not be indicative of allerginicity at all. Duarte et al. (2007) studied the increase of specific IgE antibodies to *Plasmodium falciparum* in infected patients. The *P. falciparum* specific IgE response did not correlate with anti-inflamatory cytokin patter bias during malaria but rather seemed to contribute to the control of parasites, since functional activity was higher in asymptomatic and uncomplicated malaria patients than in severe or cerebral malaria groups. Bereczky et al. (2004) found the same sort of response where elevated *P. falciparum* specific IgE levels reduced the change of presenting clinical malaria.

IgE induction by Bt

Laferrire et al. (1987) reported that forest spray workers who used Btk had a significant elevation in antibody titers and levels were higher in workers who were exposed for 2 years. Antibody titers reduced rapidly after exposure ceased and thus the probability that this would result in clinically defined allerginicity in these workers is low. The class of Btk antibodies is not reported so allerginicity is also not clear. Only 5/112 workers were reported to be positive for the antibodies. The study did not report any exposure-related clinical manifestations in the workers.

A longitudinal, follow-up investigation of 48 workers who were involved in picking Bt (Javelin®) sprayed crops (celery, parsley, cabbage, kale, spinach, strawberries) was conducted by Bernstein et al. (1999). Bernstein presented three exposure groups: "Low" (handled onions not Bt sprayed 3 miles away), "Medium" (packaged Bt treated crops) and "High" (picked Bt treated crops). There was no evidence of occupationally related respiratory symptoms. Ocular and dermal symptoms occurred across the three groups and appear to be related to crop exposure reactions and not Btk. Positive skin tests to several spore extracts were seen, chiefly with exposed workers (35%). Yet the "Low" exposure group presented at 25% for an atopic response. Positive skin test were seen with water and mercaptoethanol-sodium dodecyl sulfate extracts of Javelin, yet did not increase for Javelin extracted pro-Delta-endotoxin and protenaise K spore extracts. Specific IgE and IgC antibodies to vegetative cells were present in all groups, which could bring up the question of whether these antibodies were possibly reacting to some cross reactivity.

Doekes et al. (2004) tested sera from the BIOGART project, a longitudinal respiratory health study on >300 Danish greenhouse workers. This study had a 2 and 3 year follow up. While many sera had detectable IgE to Bt (23-29%), all positive reactions were relatively weak IgE anti-Bt positive sera showed, with one exemption, only OD492 values <0.2. These highly exposed workers presented no evidence for work related respiratory symptoms, even after numerous years of exposure. The authors conclude that even while the IgE binding components may be genuine Bt components, they could also be shared with some or many commonly found bacterial species, and in that case the IgE levels may be found in the general population.

What sensitization would be expected from airborne microbials: Asthma?

Even Burrows et al. (1998), who showed a relationship between IgE levels and asthma, pointed out that "although pneumococcal-specific IgE can sometimes be demonstrated, there is no firm evidence that allergy to bacteria in the airway is of importance in the pathogenicity of asthma." Asthma is a heterogeneous condition with multiple biological unique etiologies and is considered to involve allergic and non-allergic mechanisms.

As recommended by Burrows et al., clusters of asthma within occupational groups appear to be a good way to determine allergic stimuli. This is how swine dust, cotton dust, etc. have been identified as allergens. Yet, in the 30+ years that Bts have been extensively used, these asthma clusters have not been seen.

Several studies have in fact shown that exposure to bacterial lipopolysaccharide can either prevent or inhibit asthma in humans or laboratory rodents. While the mechanism of the effect is open to further research and several hypothesis have been put forward, the actual down modulation to antigen induced asthma is well documented (Lundy et al.2003). It is therefore doubtful that at the exposure levels experienced in the field upon spray application that Bts would lead to clinical symptoms of asthma.

Epidemiological Review:

Several epidemiological studies have shown an inverse relationship between human asthma and environmental exposure to LPS (von Mutius et al. 2000; Braun –Fahrlander et al. 1999 and 2002).

The lack of Bt clinical allergenicity development is strengthened by epidemiological data because no increase in respiratory problems has been demonstrated, even when asthmatic individuals are closely monitored.

In a 2-year study, Green et al., 1990, conducted a surveillance program with the four largest medical laboratories in a Btk spray area (80,000 people in 1985 and 40,000 people in 1986). A non-sprayed community 100 km away served as a control population. There

was no increase in the number of telephone complaints nor was there a change in the pattern of complaints.

Another study was conducted (Nobel et al. 1992) in an area with a population of 1,400,000. There was no difference in emergency room visits between spray days and non spray days, nor a significant change in the pattern of telephone calls. There was no evidence that Bt was associated with illness or infection and, in fact, there was a lower incidence of diarrhea in the spray zone than in the control area.

Similar conclusions, of no health effects, can be drawn from the epidemiological studies carried out during eradication spray programs conducted in New Zealand.

References:

Baldachi s, Carrozzi, L, Viegi G, Giuntini C., (1997) Assessment of respiratory effect of air pollution: study design on general population samples. JEPTO 16: 77-83.

BERECZKY Sandor; MONTGOMERY Scott M.; TROYE-BLOMBERG Marita; ROOTH Ingegerd; SHAW Marie-Anne; FÄRNERT Anna . (2004) Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. International journal for parasitology (Int. j. parasitol.) , vol. 34, no8, pp. 935-942

Braun-Fahlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, vuille JC, Wuthrich. (1999) Prevalence of hay fever and allergic sensitization in farmer's children and their pers living in the same rural community. SCARPOL team. Swiss Study on Childhood allergy and Respiratory symptoms with respect to Air Pollution. Clin. Exp. Allergy 29: 28 – 34.

Braun-Fahlander C, Riedler J, Hertz U, Eder W., Waser M. Grize L., Maisch S, Carr D, Gerlach F, Bufe A, Launer RP, Schierl R, Renz H, Nowak D, von Mutius E.(2002) Environmental Exposure to endotoxin and its relation to asthma in school-age children. N. Eng. J. Med. 347: 869-877.

Burrows B, FD Martinez, M Halonen, RA Barbee, and MG Cline. (1989) Association of asthma with serum IgE levels and skin-test reactivity to allergens. New England Journal of Medicine Volume 320:271-277

Ciprandi, G; A. Vizzaccaro, I. Cirillo, M. A. Tosca (2005) *Bacillus clausii* effects in children with allergic rhinitis. Allergy 60 (5), 702-703.

Doekes, G, Preben Larsen, Torben Sigsgaard, and Jesper Baelum. (2004) IgE Sensitization to Bacterial and fungal Biopesticides in a Cohort of Danish Greenhouse Workers: The BIOGART Study. American Journal of Industrial Medicie 46: 404-407.

Douwes J and Pearce N (2002) Asthma and the westernization 'package'. International Journal of Epidemiology 31: 1098 – 1102.

Duarte J, Desshpande P., Guiyedi V. Mécheri S, Fesel C, Cazenave P-A, Mishra GC, Kombila M, Pied S. (2007) Total and functional parasite specific IgE response in Plasmodium falciparum-infected patients exhibiting different clinical status. Malaria journal 6: 1- 13

Erb KJ, Holloway JW, Sobeck A., Moll H. Le Gros G. (1998) Infection of mice with Mycobacterium bovis – Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinohilia. J Exp Med 187: 561-569.

Georges, C.L.S., White M.L., Kulhankova K., Mahajam A., Thorne, P.S., Snyder J.M., Kline J.N. (2006) Early exposure to nonhygienis environment alters pulmonary immunity and allergic responses. Am J Physiol Lung Cell Mol Physiol 291: L512-L522

Green MG, Heumann M, sokolow R., foster LR, Bryant R, Skeels M. (1990) Public health implications of the microbial pesticide Bacillus thuringiensis: An epidemiological study, Oregon, 1985 – 86. Am J Public Health 80: 848 – 852.

Hertz U., Gerhold K., Gruber C, Braun A., Wahn U. Renz H, Paul K. (1998) BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. J Allergy Clin Immunol. 102: 867 - 874

Institut Pasteur and Institut National de la Sante et de la Recherche Medicale, United States Patent 20070190076 (2006) Gram positive bacteria preparations for the treatment of disease comprising an immune dysregulation.

Jansen DF, Rijken, Schouten JP, Kraan J, Weiss ST, Timens, Postma DS. (1999) The relationship of skin Test Positivity, High Serum Total IgE levels, and Peripheral Blood Eosinophilia to symptomatic and Asymptomatic Airway Hyperresponsiveness. Am J Respir Crit Care Med 159: 924-931.

Laferrière, Michel. Étude immunologique impliquant les composantes de l'insecticide biologique Bacillus thurigiensis var. kurstaki : rapport 1987 / Laferrière, Michel, Bastille, Alain, Nadeau, Aubert [i.e. Michel Laferrière, Alain Bastille, Aubert Nadeau]. -- Rivière-du-Loup, Québec : Dép. de santé communautaire du Centre hospitalier régional du Grand-Portage, 1987.

Lundy, S.K., A..A Berlin N.W. Lukacs (2003) Interleukin-12-Independent Down-Modulation of Coackroack Antigen-Induced Asthma in Mice by Intranasal Exposure to Bacterial Lipopolysaccharide. American journal of Pathology vol.163; 1961-1968.

Nobel MA, Riben PD, Cook GJ. (1992) Microbial and Epidimiological Surveillance Programme to monitor the Health Effects of Foray 48B BTK Spray. Ministry of Forests, Province of British Colombia.

PASTORELLO E. A. ; INCORVAIA C. ; ORTOLANI C. ; BONINI S. ; CANONICA G. W. ; ROMAGNANI S. ; TURSI A. ; ZANUSSI C. Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy. I: Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens. Journal of allergy and clinical immunology (J. allergy clin. immunol.) 1995, vol. 96 (1), n°5, pp. 580-587.

Rylander, Ragnar. Review: Endotoxin in the environment – exposre and effects. (2002) J Endotoxin Res. 8:241-252.

Shida, Kan , Kumiko Makino, Aki Morishita, Kotaro Takamizawa, Satoshi Hachimura, Akio Ametani, Takehito Sato, Yoshihiro Kumagai, Sonoko Habu, Shuichi Kaminogawa (1998) Lactobacillus casei Inhibits Antigen-Induced IgE Secretion through Regulation of Cytokine Production in Murine Splenocyte Cultures
International Archives of Allergy and Immunology 1998;115:278-287

Tulić MK, Wale JL, Holt PG, Sly PD. (2000) Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide. Am. J. Respir. Cell Mol. Biolo. 22: 604 – 612.

Vojdani, Aristo; Andrew W. Campbell; Albert Kashanian; Elroy Vojdani. June, 2003 Antibodies against molds and mycotoxins following exposure to toxigenic fungi in a water-damaged building. Archives of Environmental Health,

Von Herrtzen L. and Haahtela T. 2006 Disconnection of man and the soil: Reason for the asthma and atopy eidemic? J Allergy Clin Immunol Volume 117, Number 2

Von Mutius E, Braun-Farlander C, Schieri r, Riedler J., Ehlermann s, Maisch S, Waser M, Novak D. (2000) Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp. Allergy 30: 1230-1234.

Zuany-Amorim C, Sawackia E., Manilus c., Le Moine A., Brunet LR, Kermeny DM, Bowen G, Rook G, Walker C. (2002) Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen specific regulatory T-cells. Nat. Med 8: 625-629.

Eur Respir J 2001; 17: 773–790
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

SERIES "CONTRIBUTIONS FROM THE EUROPEAN RESPIRATORY MONOGRAPHS"
Edited by M. Decramer and A. Rossi
Number 1 in this Series

Allergy markers in respiratory epidemiology

S. Baldacci*, E. Omenaas#, M.P. Oryszczyn¶

Allergy markers in respiratory epidemiology. S. Baldacci. ©ERS Journals Ltd 2001.
ABSTRACT: Assessing allergy by measurement of serum immunoglobulin (Ig) E antibodies is fast and safe to perform. Serum antibodies can preferably be assessed in patients with dermatitis and in those who regularly use antihistamines and other pharmacological agents that reduce skin sensitivity.

Skin tests represent the easiest tool to obtain quick and reliable information for the diagnosis of respiratory allergic diseases. It is the technique more widely used, specific and reasonably sensitive for most applications as a marker of atopy.

Measurement of serum IgE antibodies and skin-prick testing may give complimentary information and can be applied in clinical and epidemiological settings.

Peripheral blood eosinophilia is less used, but is important in clinical practice to demonstrate the allergic aetiology of disease, to monitor its clinical course and to address the choice of therapy. In epidemiology, hypereosinophilia seems to reflect an inflammatory reaction in the airways, which may be linked to obstructive airflow limitation.
Eur Respir J 2001; 17: 773–790.

*Institute of Clinical Physiology, Pisa, Italy. #Dept of Thoracic Medicine, University of Bergen, Bergen, Norway. ¶INSERM U472, Villejuif, France.

Correspondence: S. Baldacci, Istituto di Fisiologia Clinica, CNR, Via Trieste 41, 56126 Pisa, Italy.
Fax: 39 50503596

Keywords: Atopy, eosinophilia, epidemiology, general population, immunoglobulin E, skin test reactivity

Received: December 11 2000
Accepted after revision December 15 2000

The term "allergy" was introduced in 1906 by the Viennese paediatrician von Pirquet to point out a condition of altered reactivity in a host organism. He observed that the introduction of an external substance in a tissue could alter the tissue reactivity upon a subsequent contact with the same substance. Previously, the term "allergy" was used to define these altered responses, either protective or harmful. More recently, this term has been used to indicate an abnormal modality (hypersensitivity) of the organism to respond to antigenic stimuli (antigens) which are inactive in normal subjects [1].

In 1921, the experiments of two German physicians, C. Prausnitz and H. Küstner, demonstrated that allergy was correlated with a serum factor which was subsequently defined "reagine" [2]. In 1923 A.F. Coca and R. Cooke introduced the term "atopy" to define a constitutional status of predisposition to develop allergic diseases as pollinosis and bronchial asthma with a "reaginic" pathogenesis [3]. The term atopy, of Greek etymology, indicates clinical conditions with unusual characteristics.

For many years, few investigators tried to identify and to separate the reagines. Only in 1966–1967, in Denver, USA, two researches were able to separate, in the serum of atopic subjects, a factor with high reaginic activity, pointing out that it did not identify itself with any immunoglobulin (Ig) class known at the time. This new Ig was called "IgE" [4].

At the same time, in Sweden, two other researchers, H. Bennich and S.G.O. Johansson, identified a new Ig in a patient with a myeloma, which was defined IgND. Furthermore, they observed that high levels of IgND were present in the serum of atopic subjects [4]. The immunological and physical-chemical characterization allowed the identification of the IgND with the IgE. In 1968 in Losanna, an International Committee of experts of the World Health Organization International Reference Centre for Immunoglobulins decided to call this immunoglobulin definitively "IgE" recognizing it as the 5th antibody class.

The discovery of IgE has led to an understanding of the mechanisms of allergy. It has also led to the development of diagnostic tools as well as analyses and standardization of allergen extracts. It is possible to measure not only the pure protein content, but also the allergenicity in terms of the antigen that reacts with the IgE antibodies. The next possibility is to develop allergens for diagnostic and therapeutic endpoints including immunotherapy [5]. Advanced treatment principles of immunological modification of the functions of IgE antibodies and the mechanisms that regulate the IgE production may in the future include blocking or down regulation of IgE in patients with allergic rhinitis and asthma [5].

Atopy, with or without clinical symptoms, is an important risk factor for asthma [6], hay fever and eczema and creates interest for assessment in epidemiological studies. Clinicians tend to validate IgE antibody testing against its prediction of clinical atopic disease, while epidemiologists want to identify individuals with atopy regardless of the presence of disease. Thus criteria for test positivity in clinical and epidemiological settings may differ.

In epidemiological surveys, application of biological markers is ideally required to assess the exposure-disease relationship [7]. However, allergic sensitization, assessed as serum specific IgE antibodies, is not an

indicator of allergen exposure alone. In addition to allergen exposure, serum specific IgE antibodies reflect the individual's susceptibility for allergic sensitization as well as health effects, and may reflect more of the events in the airways when compared to skin tests thus giving additional information [8].

Skin tests represent a practicable and reproducible investigation which allows, when correctly performed, quicker and cheaper results than any other technique in the diagnosis of respiratory allergic diseases.

Skin tests give a semiquantitative measure of sensitization, as they are supposed to reflect the sensitization status of airways mucosae. In fact, using a skin test, an interaction is artificially created between IgE bound to mast cells receptors and the same allergens which are present in the atmosphere and which are spontaneously inhaled.

It is important to point out that there has been wide use of skin test reactivity in respiratory epidemiological studies on general population samples, over recent years, to improve the knowledge on the natural history of airways obstructive diseases. In fact, atopy, as assessed by skin test reactivity, is considered a risk factor for asthma and for bronchial hyperresponsiveness, which is considered the main functional characteristic of asthma [9]. However, atopic predisposition, as assessed by prick test reactions, seems to be related not only to classical allergic conditions and symptoms, but also to other respiratory problems.

In contrast to the "British hypothesis" [10], but in agreement with the "Dutch hypothesis" [10], Finnish studies showed that atopy predispose to chronic bronchitis and, furthermore, atopy and smoking seemed to have an independent and additive effect on both the prevalence and incidence of chronic bronchitis [10].

During the past 20 yrs, there has been a growing interest in the effector functions of the eosinophil granulocyte, including a variety of clinical conditions associated with hypereosinophilia, such as asthma, allergic diseases, intestinal diseases, joint diseases and skin diseases [11].

Studies of allergy and respiratory disease have traditionally used allergy skin test reactivity, serum IgE levels or peripheral blood eosinophilia to identify atopic subjects. The strong correlation between these phenotypic markers has led to the common assumption that they are more or less interchangeable indicators of the atopic state. However, recent epidemiological investigations have provided evidence that each of these markers may represent a specific risk factor for specific categories of atopic diseases [12]. In particular, peripheral blood eosinophilia has been identified as an independent inflammatory marker for symptoms of cough and phlegm, distinct from IgE or skin test reactivity [12].

Furthermore, increased serum total IgE levels and peripheral blood eosinophil counts are neither closely related nor exclusively present in atopic individuals. Serum total IgE levels are increased in nonatopic smokers and peripheral blood eosinophils are also elevated in parasitic infections and in certain neoplasms [13].

In the development of allergic diseases, an important role is played by the environmental allergens, which stimulate an immunological specific IgE response in genetically predisposed subjects. The reacting substances, which are immunologically true antigens, are generally called allergens [2]. They are heterologous proteins or glycoproteins inducing specific allergic reactions in sensitized subjects. Allergens causing atopic reactions are able to induce IgE production and, by interacting with them, to determine clinically evident manifestations. Allergens are classified according to the way of penetration into the organism: by inhalation (aeroallergens), by ingestion, by injection or by contact.

The aim of this review article is to summarize the epidemiological evidence which has emerged during the last decades relating the role of allergic markers such as serum total and specific IgE, skin reactivity and eosinophils in respiratory epidemiology.

Total and specific immunoglobulin E antibodies

Clinical and epidemiological importance

Assessment of IgE antibodies is usually performed in order to evaluate atopy. Subjects who are readily triggered to produce IgE antibodies after exposure to common environmental allergens are defined as atopic [14]. This definition excludes those who have high total IgE levels, but no specific IgE antibodies against common environmental allergen, as measured by skin-prick testing or serum specific IgE antibodies. It also excludes those who have become sensitized only to uncommon allergens [15].

The role IgE plays in immediate-hypersensitivity (allergic) reactions is well understood. Consequently, nowadays IgE measurements in serum are performed more frequently as part of routine allergy testing. They are easy to perform, and new methods and equipment make them less expensive to use. Furthermore, external quality assurance schemes are routinely used and results of testing are likely to be identical irrespective of where they have been performed. Use of long-acting antihistamines will not affect the results of serum examinations, while it can reduce skin tests. The relationship between the clinical history and IgE antibody testing is not always quantitative in the clinical setting. Likewise, not all symptoms associated with allergic disease are directly related to IgE-mediated hypersensitivity reactions, for example intrinsic asthma, drug and food intolerance. Consequently, it is advisable to ensure that symptoms are genuinely provoked by an allergic mechanism before undertaking allergy treatment, which will be time consuming, expensive and demanding good collaboration between patient and doctor.

The large range of normal adult IgE levels results in a considerable overlapping of normal and atopic ranges and, consequently, the test cannot identify the atopic person. In a recent published textbook [8] circulating levels <20 $kU \cdot L^{-1}$ render a diagnosis of atopy unlikely, while concentrations >180 $kU \cdot L^{-1}$ indicate a likely diagnosis of atopy. However, there is significant variation by sex, age, smoking habits and geography which may indicate that local reference values with proper adjustments should be applied [16]. Furthermore,

markedly raised IgE levels have been reported in a variety of other conditions, including parasitic disease, Wiskott-Aldrich syndrome, Job-Buckley syndrome, alcoholism, human immune deficiency virus (HIV) syndrome, pemphigoid and occasionally, Hodgkin's disease as well as after severe burns [8]. As a rule, total IgE levels among atopics correlate with the size of the target organ, so the lowest levels are observed among rhinitics, the highest in those with atopic eczema, and the intermediate in asthmatics [8].

An elevated total IgE is more likely to be correlated with multiple positive specific IgE (RAST) tests than is a normal total IgE [8, 17]. Levels >5,000 $kU \cdot L^{-1}$, which often occur in cases with severe atopic eczema, are a warning that artefactually high results may have occurred in the IgE antibody test. Although a total IgE estimation is not a good diagnostic test to differentiate atopy from normal, when taken together with the clinical history it allows a more complete interpretation of the available data.

The routine use of IgE antibody tests should be restricted to diseases in which the role of IgE has been well documented, such as allergic rhinitis, bronchial asthma, atopic eczema, food, venom- and drug-induced anaphylaxis and bronchopulmonary aspergillosis [8].

Normal values of IgE are usually very low, but people with hereditary or constitutional predisposition to allergy (atopy) produce relatively greater quantities of IgE. In addition, the production of IgE antibodies can continue for years after a contact with an allergen. High levels of total serum IgE in humans have been associated with wheeze, hay fever, asthma and eczema.

An increasing array of blood tests is available to measure specific IgE in serum directly. A positive skin test relies on both presence of specific allergic reactions as well as unspecific reactions in the skin, which can promote degranulation of sensitized mast cells. The lack of agreement between skin and serological tests may reflect differences in the presentation of allergen and/or the presence of non-IgE mediated skin reactions.

Prevalence in epidemiological studies

Observed variation in the prevalence of atopy and atopic disease may reflect a true variation. However, this may partly be explained by variation in the prevalence of the recognized risk factors, which should always be taken into account.

There is some knowledge of total serum IgE levels in selected and general populations, while little information is available on prevalences of serum specific IgE antibodies due to higher costs of the examination than for skin testing. Total IgE in a population is normally distributed after logarithmic transformation, with a relatively small proportion of individuals having levels of total IgE below the limits of detection of the test (2 $kU \cdot L^{-1}$). The geometric mean of total serum IgE as well as specific serum IgE was lower in Nordic populations [17, 18] than in young adults in South Wales [19], adults in Tucson, Arizona [20], in Italy [21] and in the Netherlands [22]. This may partly be due to differences in methods, in mean age of the populations, as well as differences in allergen exposure. In the European Community Respiratory Health Survey (ECRHS) [16], the prevalence of atopy, assessed as serum specific IgE antibodies, was lowest in young adults in the Nordic countries and highest in those living in the UK, New Zealand and Australia (fig. 1) [16]. Thus, serum specific IgE levels vary with area of residence possibly reflecting differences in allergen exposure and in individual susceptibility. There have been few studies documenting any change in the prevalence of atopy assessed with specific IgE antibodies within populations. Two small studies have reported an increase of atopy in children [23, 24].

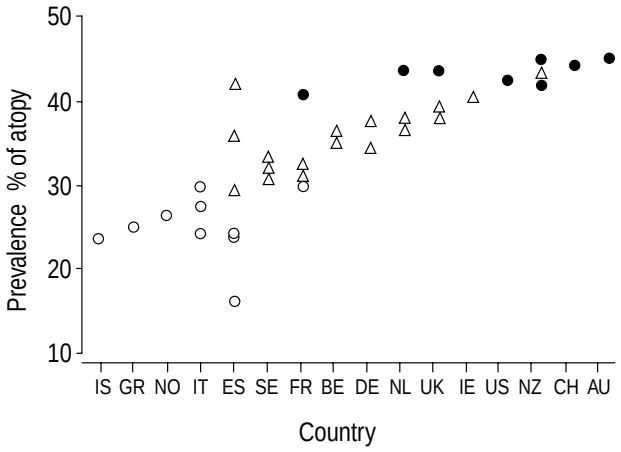


Fig. 1. – Prevalence of atopy in young adults, defined as specific immunoglobulin (Ig)E to any of the five allergens tested in each centre plotted by country and centre in the European Community Respiratory Health Survey. Each symbol represents the value for one centre. The countries are ordered along the X-axis. Values significantly above or below the median for 35 centres are indicated. The countries are: IS: Iceland; GR: Greece; NO: Norway; IT: Italy; ES: Spain; SE: Sweden; FR: France; BE: Belgium; DE: Germany; NL: Netherlands; UK: United Kingdom; IE: Ireland; US: United States; NZ: New Zealand; CH: Switzerland; AU: Australia. Adapted from reference [16]. ○ : 95% confidence interval (CI) below and excludes study median; △ : 95% CI includes study median; ● : 95% CI above and excludes study median.

A positive relationship has been reported between total serum IgE level and presence of serum specific IgE antibodies in clinical settings, in selected populations as well as in a general population [17]. The predictive value of having one or more serum specific IgE antibodies given total serum IgE level in the highest quintile is nearly doubled in males compared to females, in younger compared to older subjects and in nonsmokers compared to smokers [17]. However, this was not observed in young adults in the ECRHS [16].

Allergy and skin test reactivity

Clinical and epidemiological importance

An accurate diagnosis of allergy precedes the effective management. Allergy tests are used to define the subset of subjects who, because of sensitivity to allergens, have symptoms caused by one or more allergens, and to identify the involved allergen or allergens.

Specific sensitization assessment should always be performed before allergen eradication procedures, in

order to implement an appropriate therapy for any particular individual [25]. On the contrary, negative results of allergy tests can discriminate subjects whose symptoms are not caused by allergens from subjects with allergen-mediated disease. Skin tests are *in vivo* allergy tests used to detect immediate (antibody-mediated) or delayed (cell-mediated) sensitivity. They were first used over a century ago to confirm the association between hay fever and pollen sensitivity [2]. The delayed cutaneous response is defined as the skin erythema and induration produced by an intradermal test 6 h after applying the test.

Skin-prick testing represents an inexpensive, quick, safe diagnostic procedure for allergy, but, in order to obtain reliable clinical results, a few rules must be followed. The allergen potency must be kept constant by use of freeze-dried extracts, stored at $\leqslant$-10°C [26]. Dilutions of the concentrated extract must be accurately prepared. Considering prick tests, 50% glycerine is used to preserve dilutions, while intradermal test extracts are diluted in HSA-saline (normal saline with 0.03% human serum albumin preserved with 0.4% phenol).

Patients must avoid drugs that inhibit the immediate cutaneous response such as astamizole for $\geqslant$6 weeks; hydroxyzine, ketotifen, and tricyclic antidepressants, like imipramine, for $\geqslant$2 weeks; conventional antihistamines and oral or injected adrenergics for $\geqslant$72 h [26]. In addition, test results may be misleading in skin disorders such as dermatographism and generalized eczema. It is better to do skin tests at the same time of the day to minimize circadian effects on the response.

The number of allergens used in clinical practice has been increasing and includes extracts derived from many pollens and moulds, various dusts and insects, animal pelts, foodstuffs and numerous bacterial vaccines. In recent decades, a patient having an allergy evaluation might be theoretically tested for as many as 300 allergenic substances.

Skin-prick tests have been commonly chosen to measure atopy in populations, largely because the procedure is simple and well documented. However, the criteria by which atopy is defined are not well established: the minimum skin weal size used to indicate atopy remains controversial and the number of allergens required to detect all atopic subjects is unknown [27]. Indeed, because of the wide number of potentially allergenic substances and variations in local allergen levels, the number of allergens in a screening panel could be very large, although in a few countries, such as in the UK and in Sweden, mini-panels of four selected allergens detect most atopy [27]. Finally, although skin-prick tests have been shown to correlate well with specific serum IgE, recent data indicate that the presence of skin-prick test reactivity does not have the same meaning as increased level of total serum IgE; thus, in epidemiological studies, it is advisable to use both allergy markers, in order to have a complete and precise evaluation of the immunological status [12].

Prevalence in epidemiological studies

In the last 15 years, two Italian general population samples, selected according to standardized and adequately defined procedures, were investigated and followed over time [28]. They live in two different areas, a rural area in Northern Italy (Po river Delta) and an urban area in Central Italy (Pisa). The prevalence of respiratory symptoms was higher in the urban area compared with the rural area. Conversely, no difference between the rural and urban areas was evident when comparing skin reactivity prevalence as a marker of atopy: 31% in the rural area compared to 32% in the urban area [9, 28].

Table 1 shows the prevalence of skin test reactivity in different studies and populations: random samples of communities, subjects free of allergic symptoms or allergic populations. A wide range of prevalence rates of skin-prick reactivity (21–49%) has been reported from studies on general populations or subjects free of allergic symptoms. Studies on allergic populations have reported prevalences >50%.

Possible causes of this large variability reported in the literature, include the characteristics of the populations observed, the number and the potency of allergenic extracts, and the criteria for evaluating of skin reaction.

Table 1. – Prevalence of skin test reactivity in different epidemiological studies

Author	[Ref.]	Population	Prevalence of skin reactivity %
Children - Adolescents			
HAAHTELA	[29]	708 Finnish, 15–17 yrs	49
Adults			
BARBEE	[30]	US community sample of 3012 subjects, 3–75 + yrs	34
BALDACCI	[28]	2,184 subjects participating in the Pisa survey, 8–84 yrs	32
BALDACCI	[9]	2,632 subjects participating in the Po Delta survey, 8–75 yrs	31
TOLLERUD	[12]	1,071 males participating in the Normative Aging Study, 21–80 yrs	21
SIN	[31]	277 young healthy individuals in Izmir, Turkey, 15–25 yrs	46
NOWAK	[32]	1,890 subjects participating in the ECRHS survey, in West and East Germany, 20–44 yrs	East 30 West 36
HERXHEIMER	[33]	300 subjects with respiratory allergies in London, 5–75 yrs	95
FREIDHOFF	[34]	262 subjects reporting allergies in Baltimore, 18–55 yrs	55
SIBBALD	[35]	1,305 rhinitic subjects in Southwest London, 16–65 yrs	59
BOULET	[36]	3,371 allergic patients in Canada, $\geqslant$16 yrs	80

Generally, in epidemiological studies, the choice of representative samples, randomly selected and stratified for sex, age, socioeconomic status or other discriminating factors, allows inference to be made on the general population living in a certain area.

The potency of allergenic extracts is one factor that may account for discrepancies between different studies; nevertheless, the use of new extraction methods in recent epidemiological studies should have reduced these differences.

The most important sources of variability among different epidemiological studies might be different criteria of reading skin reactions, such as different size of reaction or different modalities of reading (maximal with or without minimal diameter, area, *etc.*). Finally, the use of different allergens (qualitatively or quantitatively) may constitute an additional source of variability [37].

With regard to specific skin responses, epidemiological studies indicate that Dermatophagoides, Parietaria, and Graminaceae are the allergens which most frequently give positive reactions in Italy [9, 38].

In an Australian study [27] the prevalences of sensitization, investigated in three populations of schoolchildren living in different climatic areas, were higher for house dust mites in Belmont and Villawood, and for pollens of plantain, timothy grass, rye grass and *Aspergillus fumigatus* in Wagga Wagga. The prevalence of animal dander sensitization was low in all areas.

SIN *et al.* [31] reported that sensitization to house dust mites was most frequent in Turkish subjects aged 15–25 yrs.

Eosinophils

Clinical and epidemiological importance

Eosinophils release specific lipid mediators, including leukotriene C4, platelet-activating factor (PAF) and lipoxins, and contain four different cationic proteins within their granules: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin, which are characterized by a strong cytotoxic potential [11].

The eosinophil is derived from the common haematopoietic stem cell. The most important growth factors involved in eosinophil maturation and proliferation are granulocyte-macrophage colony stimulating factor, interleukin-3 and interleukin-5. The normal concentration of eosinophils in the blood is 1–4% of the leukocytes, while it is as high as 100–300 times in tissues, suggesting that the eosinophil should be mainly regarded as a tissue cell. In the nasal secretion of subjects with rhinitis the eosinophils concentration may also reach very high levels, accounting for $>90\%$ of all the recovered cells. The half-life of eosinophil in the blood is 4–5 h, while the half-life in tissues is 8–12 days [39].

In clinical practice, peripheral blood eosinophil counts are widely used to demonstrate the allergic aetiology of disease, to monitor its clinical course and to address the choice of therapy [40]. Moreover, this parameter seems to be an early atopy marker since elevated eosinophil count in peripheral blood of apparently healthy infants at 3 months of age is associated with a subsequent diagnosis of atopic disease [41].

The diagnosis of atopic disease is often difficult in small children because of differences in symptoms and lack of specific and reliable laboratory tests. A study, evaluating the significance of four commonly used laboratory tests (blood eosinophil counts, total serum IgE and eosinophil and mast cells in the nasal smear) as atopy markers in 178 children aged 3 yr, showed that a blood eosinophil count $>600 \times 10^3 \cdot \mu L^{-1}$, as well as an increased number of eosinophil and mast cells in the nasal smear, were associated with atopy. However, normal levels of these parameters did not exclude atopic disease. Thus, each of these tests had high specificity, but low sensitivity [42].

Antigens (*via* antigen presenting cells) can activate T-helper cells, and subsequent lymphokine production. Some of these lymphokines (interleukin-4, interleukin-5 and interferon gamma) can activate B lymphocytes, which are in turn responsible for IgE secretion. Moreover, T-helper cells-derived lymphokines are also known to be associated with higher eosinophil counts. As a consequence, allergens can trigger both IgE synthesis and eosinophil proliferation. However, not only allergic stimuli, but also nonallergic stimuli (especially cigarette smoking) are known to be associated with higher eosinophil counts, possibly *via* T-cells. Eosinophil proliferation might, therefore, be the result of either allergic and/or nonallergic stimulation [40].

Atopy might be one of the pathophysiological mechanisms responsible for the development and evolution of COPD. Both host and environmental factors, which play an essential role in determining atopy, may influence the risk for the development and evolution of COPD. Atopy may induce or contribute to the development and progress of COPD in many ways, including nonallergic mechanisms. Epidemiological findings have shown that blood eosinophil count appears inversely related to the level of pulmonary function and directly related to the rate of decline of pulmonary function among nonsmokers [43, 44]. In particular, a link of either inflammation (hypereosinophilia alone) or allergic inflammation (eosinophilia in combination with skin test reactivity) with level of forced expiratory volume in one second (FEV_1), independent of the effect of cigarette smoking, was found [44]. Furthermore, elevated blood eosinophilia has been found significantly related to several respiratory symptoms and disease diagnoses [12, 45]. Therefore, on the epidemiological side, peripheral blood eosinophil count can be useful both to investigate the association of host factors and environmental determinants to indicators of allergy prevalence and to evaluate whether allergy is a risk factor with a possibly substantial contribution to the development and progress of either reversible and irreversible airflow obstruction.

Prevalence in epidemiological studies

The use of different measures of atopy in different studies has complicated the comparison of the results.

In particular, as regards peripheral blood eosinophils, there are only few epidemiological studies including this atopy marker on a large scale, and, to add further complexity, eosinophil count could be considered both as a continuous and/or as a categorical dichotomous variable.

MENSINGA *et al.* [44] found a prevalence rate of eosinophilia (*i.e.* $\geqslant$275 eosinophils·mm^{-3}) of 12.9% (14 in males, 11.7% in females) in the general population sample of Vlagtwedde-Vlaardingen investigated in the initial and in the first follow-up surveys. Further, the analyses made using the data from the subsequent follow-up surveys of the same longitudinal population study, showed prevalence rates of peripheral blood eosinophilia slightly higher (15.3%), comprised between the prevalence of skin test positivity (14.7%) and of increased serum total IgE level (16.9%). The overlap among the three measures was small [13].

In the Normative Aging Study [12], which has analysed the relationship of respiratory symptoms with the three main phenotypic markers (skin test reactivity, serum Ige, peripheral eosinophils), the mean values of eosinophils have been reported both as cells·mm^{-3} (207±5 in nonsmokers, 268±15 in current smokers) and as percentage on total white blood cells (3.7±0.1 in nonsmokers, 3.9±0.2% in current smokers).

Conversely, TAYLOR *et al.* [46], in a study concerning the association between smoking itself and indicators of allergy, reported lower values of the percentage of eosinophils, 1.67% in nonsmokers and 1.95% in smokers.

ULRIK [43], in a study on a population sample of 665 adolescents and young adults, aged 13–23 yrs, reported a prevalence rate of eosinophilia (defined as >0.25 cells $\times 10^3 \cdot \mu L^{-1}$) of 13.2% in a subgroup of nonsmoking, nonatopic, nonasthmatic subjects with no evidence of bronchial hyperresponsiveness.

In conclusion, these findings support the theory that the role of the eosinophils in obstructive pulmonary diseases extends beyond its role in allergic reactions, suggesting that an increased number of blood eosinophils reflects an inflammatory reaction in the airways, which might cause the development of obstructive airflow limitation.

Modifiers of allergy markers

Allergy markers are affected by various factors, both endogenous and exogenous, and many pieces of evidence exist nowadays due the conduction of epidemiological studies. Besides well-known factors like smoking [47], additional factors have recently been suggested as potential modifiers of allergy markers but they need to be investigated further. Results are more consistent in the case of total IgE level due to the fact that IgE assessment is largely reliable. In the case of skin prick tests, the lack of standardization on the method of performing the tests has prevented generalization of results. In human beings, eosinophils intervene in atopy and eosinophilia, including circulating eosinophilia, is associated with asthma and with bronchial hyperresponsiveness [48]. Modifiers of eosinophilia have rarely been studied. In order to avoid biases potential modifiers need to be taken into account. Table 2 presents main modifiers of total IgE, SPT and eosinophilia respectively, according to population-based data.

Total immunoglobulin E: relationship to sex, age, ethnic group and environmental factors

Measurements of total IgE allow some quantitative assessment of genetic phenotype. Total IgE has been linked to chromosome 5q, the site of many interleukines, in pedigrees of parents with asthma [49]. When defining subjects with high total serum IgE or a positive specific IgE to a common inhalant allergen, linkage to chromosome 11q has been observed [50], possibly more important when carried by the mother. HANSON *et al.* [51] showed that twins reared apart had a high concordance of total serum IgE, but not to specific allergens when measured by RAST. These observations suggest that total IgE is largely genetically determined, while sensitization may be largely environmentally determined. As much as 50% of the variance in total IgE can be determined by genetic factors [52, 53]. Also specific IgE may to some degree be under genetic control [54].

Total IgE are higher in males than in females but this is not always observed at birth [55, 56]. The differences have been shown to be greatest in young adults, although, other studies have observed higher total IgE levels in elderly males compared to females after taking into account age, smoking habit, occupational exposure and presence of respiratory symptoms and disease [17, 57, 58]. The reason for this sex difference has not been satisfactorily explained. One hypothesis could be that a higher proportion of elderly males than elderly females have been more heavily or widely occupationally exposed, while this is not the case in younger age categories, thus representing a cohort effect.

Most reports suggest that males, in adolescence and adulthood, are at an increased risk of sensitization than females, when measured as presence of serum-specific IgE antibodies or a positive skin test [17, 59, 60]. This sex difference may be limited to some allergens as no significant sex difference for antibodies against birch and timothy was observed in a Norwegian adult population [17].

Total IgE levels depend on age but the exact relationship of dependence is not known [61, 62]. Total IgE are undetectable at birth except in allergic individuals. Among Greek children aged 1 month–14 yrs, total IgE levels increased by almost 80% per yr until 5 yrs of age [63]. Among children in the USA, followed-up from birth to 4 yrs of age, the major increase was observed before the age of 2 yrs, although total IgE level increased even later [56]. Cross-sectional data suggest that total IgE levels are at their highest in childhood and fall steadily after the age of 15 yrs [16, 21, 22, 61]. Longitudinal data from a 20-yr follow-up of people living in Tucson, Arizona largely confirm this. There is a rapid decline up to 30 yrs and thereafter the rate of decline gradually slows down [57]. A lack of decline with age, however, has been

Table 2. – Potential main modifiers of allergy markers according to population-based data

Factors	Total IgE	SPT Positivity	Eosinophils	Comments
Genetics				
Genetic markers	+	+	+	Problematic for geneticists
Gender	+	+	±	↑ IgE for men ↑ SPT for men
Ethnic group	+	+	±	↑ IgE in some groups (genetic, environmental, social)
Individual susceptibility	+	+	?	↑ IgE, ↑ atopy
Family history of atopy	+	+	?	
Individuals				
Maternal age at birth	+	?	?	↓ IgE in newborns of young mothers
Perinatal complications	±	?	?	↑ IgE in newborns
Medication during pregnancy	+	?	?	↑ IgE in newborns (progesterone)
Head circumference	±	?		↑ IgE in adults and newborns if head circumference at birth $\geqslant$37 cm
Birth weight	±	?	-	↓ IgE in low birth weight
Birth month	+	?	-	↑ IgE during pollen season
Age	+	+	+	Increase in youth and decrease with age
Infections	±	±	-	Depend on age of infection
Family size	-	+	-	Depends also on birth order
Environmental				
Maternal smoking during pregnancy	±	?	?	Contradictory results for IgE
Active smoking	+	Inversely related	+	↑ IgE with active smoking ↑ eosinophils
Passive smoking	±	±	±	Among children
Allergens exposure	+	+	-	
Month of test	±	?	-	↑ IgE during pollen season
Indoor pollution (humidity, mite)	±	±	?	↑ IgE mites
Outdoor pollution (NO_2, SO_2, diesel)	±	±	?	↑ IgE with diesel
Occupational exposure	+	+	?	↑ IgE in individuals exposed to allergens, dust, gas and fumes

?: No evidence, investigation is required; ±: uncertain relationship; +: established relationship; -: not yet studied in population-based samples; ↑: increase; ↓: decrease; SPT: skin prick test; NO_2: nitrogen dioxide; IgE: immunoglobulin E.

shown in studies including only males [12, 64], in a nonatopic population [65] and in an adult general population [17]. A lack of fall of the total serum IgE levels by increasing age in males can be due to a cohort effect, where older males were more frequently exposed to tobacco smoke and occupational exposure than younger males. Furthermore, adjustment for smoking and occupational dust or gas exposure in the statistical analysis may be incomplete.

A cross-sectional survey among Norwegian adults showed a higher prevalence of serum specific IgE in younger than older adults in both males and females. Whether this substantial fall represents a true age effect or if it is a cohort effect is still uncertain and should be addressed in follow-up studies.

Ethnic factors also influence serum total IgE levels since Black and Metis individuals have higher total IgE levels than White individuals [66]. The phenomenon begins at birth [67], probably due to helminthic infestations in such ethnic groups. This has not been confirmed by other studies. More recently, antenatal and perinatal factors have been proposed as potential modifiers of the total IgE response. Head circumference was larger in children with higher levels of cord blood total IgE [68] and serum total IgE [69]. Head circumference has been related to foetal disproportionate growing and so to deregulated immune function responsible for an over production of IgE. Similarly, alcohol and caffeine consumption by the mother during pregnancy were associated with elevated cord blood total IgE level. Such product could modify the immune response of the organisms. Among endogenous factors related to total IgE in females, there is the menstrual cycle with lower values of serum total IgE in the periovulatory phase [70].

Various exogenous factors are likely to affect total IgE level. Total IgE level varies between countries. In the ECRHS [16] the same methods for total serum IgE testing have been performed in multiple centres in Europe, Oceania and North America. Total serum IgE levels (fig. 2) were lowest in young adults in the Nordic countries and highest in southern Europe, Australia and New Zealand [16]. This may be mainly due to environmental, socioeconomic and cultural factors. There is a positive relationship between the cumulative exposure to an allergen and the risk of allergic sensitization [71]. PLATTS-MILLS *et al.* [72] suggested that there is a level below which sensitization for genetically predisposed individuals is unlikely. At higher levels of exposure, more people will become sensitized [73]. But there is a variation depending on the physical and chemical properties of the allergens. For example, cat allergen is airborne on smaller particles and remains airborne longer [72]. Currently, airborne measurements of exposure are considered too complex for epidemiological studies [72, 74].

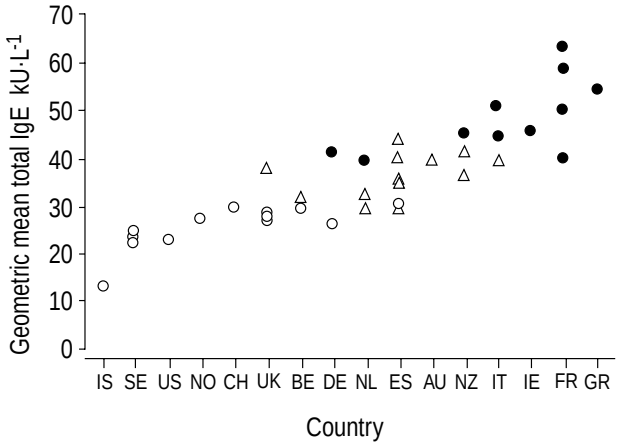


Fig. 2. – Geometric mean total serum IgE ($kU \cdot L^{-1}$) in young adults plotted by country and center in the European Respiratory Health Survey. Each symbol represents the value for one center. The countries are ordered along the X-axis. Values significantly above or below the median for 35 centres are indicated. The countries are: IS: Iceland; SE: Sweden; US: United States; NO: Norway; CH: Switzerland; UK: United Kingdom; BE: Belgium; DE: Germany; NL: Netherlands; ES: Spain; AU: Australia; NZ: New Zealand; IT: Italy; IE: Ireland; FR: France; GR: Greece. ○ : 95% confidence interval (CI) below and excludes study median; △ : 95% CI includes study median; ● : 95% CI above and excludes study median. Adapted from ref [16].

Exposure to allergens may not only increase the risk of sensitization, but may also trigger episodes of disease of those sensitized. There is no consistent evidence that exposure to allergen has increased during the last decades [73, 75]. Increases have been observed in two towns in Australia [76]. However, the trend towards better insulated houses with increases in soft furnishing, dampness and reduction in air renewal may influence the level of airborne allergens in the indoor environment [74, 77]. Some studies show increased risk of sensitization of house dust mite allergen and pets [73, 78], while others do not [79].

Month of birth during pollen season may affect cord blood IgE and later in life serum IgE [63, 80]. Both outdoor and indoor pollution exposure might be responsible for increased total IgE level. Children living in polluted areas had higher total IgE level compared to those living in unpolluted areas [81]. Atopy, assessed as skin test positivity, is highest in children who come from small families, with a linear decrease in risk of positivity with increasing number of siblings [82, 83]. On the contrary Wjst *et al.* [84] reported that total IgE levels are higher in children who come from large families, independently of exposure to passive smoking. These observations suggest that some environmental factors associated with large family size protects against the development of atopy and atopic disease. It has been postulated that higher rates of infection in children from large families, and particularly in those with older siblings, may protect them from the subsequent development of atopy by inhibiting the development of Th2-type cells. Furthermore, immunization [85] during childhood and natural infections [86] may have similar effects on the immune system.

Environmental factors such as smoking may modify the gene expression of atopy and atopic disease. The tendency for those with atopic disease to avoid smoking, the "healthy smoker" effect, may raise methodological problems when assessing the relationship between smoking and disease. Cross-sectional studies have shown higher IgE levels in smokers than in nonsmokers [17, 18, 21, 22, 87]. In a longitudinal study with a 20-yr follow-up [57] smoking was associated with a reduction in the rate at which total IgE fell with increasing age in people without atopy (skin test positivity) and with an increase in total IgE after middle age in those with atopy. This has, however, not been confirmed with serological measurements excluding bias by skin reactivity changes with age.

Only one report from a nondiseased population has observed a dose-response relationship between smoking and IgE levels [64]. An explanation for higher total serum IgE levels in smokers than nonsmokers may be change in regulatory mechanisms of the IgE synthesis [88] or increase of specific IgE related to frequent infestation of their airways [89]. It has been excluded that Th1 in response to bacterial infestations could be responsible for the augmentation of IgE. Th2 cells seem to be involved in the process. There has been no evidence of induction of specific IgE antibodies against tobacco in smokers.

A higher prevalence of serum specific IgE antibodies against timothy grass and birch in nonsmokers compared to smokers has been observed in a general adult population [17] as for grass and cat allergens in England [62]. One hypothesis often given, is that subjects with allergic symptoms and disease take up smoking less frequently or quit more often than subjects with no allergic symptoms and disease. However, this has never been shown in a prospective study. Another explanation for the lower levels of serum specific IgE antibodies in smokers compared to nonsmokers may be a defective antigenic presentation to immunocompetent cells in the airways [90].

Occupational studies [91, 92] have shown increased rate of sensitization to occupational allergens such as coffee-bean dust, phthaloic anhydride, soyabeans and prawn antigens in those who smoke.

Data existing on this topic are reviewed in table 3. Increased levels of cord IgE in newborns of mothers who smoke have been reported in some [55], but not in all studies [96]. Children from atopic families with one or more smoking parent have higher IgE than those of parents who do not smoke [97]. The relationship between maternal smoking and sensitization still remains unclear as some studies have observed no relationship [98], while others have observed a relationship in male children only [99]. Also in adults passive smoking has been associated with increased total IgE levels [21].

An exhaustive review of the literature on the effect of parental smoking on atopic status in childhood has been recently proposed [100].

Although total serum IgE levels are stable in adulthood [101] subjects with occupational dust or gas exposure in the general population had a higher total serum IgE level than those not exposed, independent of sex, age and smoking habit [17, 21]. No relationship between serum specific IgE antibodies and occupational

Table 3. – Relationship between smoking habits and total immunoglobulin (Ig)E according to recent studies in the general population

Author	[Ref.]	Subjects	Findings	Comments
BACKER Denmark, 1992	[93]	508 subjects aged 7–16 yrs	No relationship between active smoking and IgE in univariate analysis	Active smoking increased IgE after adjustment for age, SPT positivity, allergic symptoms and family history of allergic disease
JENSEN Denmark, 1992	[65]	297 smoking volunteers and 137 random controls at follow-up of: 1 yr; mean age: 44.2 yrs	Higher IgE among smokers than among nonsmokers in both sexes. Weighted pack-yr consumption associated with increased IgE	Quitters had increased IgE levels during the first 26 weeks of abstinence. Return to baseline after 1 yr
SHERRILL USA, 1994	[57]	General population: 2,350 subjects seen at least 1 time during follow-up, spanning up a period of 20 yrs, aged >6 yrs at the first survey (1973)	Higher IgE among smokers than nonsmokers in both sexes. Positive association between pack-yrs and level of IgE, stronger in male subjects	No significant difference of IgE between sexes in nonatopic subjects
OMENAAS Norway, 1994	[17]	1,512 subjects in four groups - asthmatics - nonasthmatics exposed at work -nonsmokers free of respiratory symptoms - general population aged 18–73 yrs	Higher IgE among smokers than nonsmokers	Subjects examined during the summer tended to have higher IgE level than those examined in winter. Subjects with occpuational dust or gas exposure had a higher total IgE level than unexposed. Levels of total IgE did not differ significantly by educational level
JARVIS England, 1995	[62]	General population: two samples drawn from ECRHS aged 20–44 yrs: 367 subjects, free of respiratory symptoms, 135 subjects symptomatic but without asthma before age 14 yrs	Higher IgE among smokers than nonsmokers. Difference greater in females but not significant. Weaker but positive associations between pack-yrs and level of IgE. Higher IgE among smokers than nonsmokers	In young adults, smoking explains little of the variation in total IgE (<1%)
LAI China, 1995	[94]	General population: 195 subjects among 2,032 aged $\geqslant$70 yrs	Higher IgE among smokers than nonsmokers	
WüTHRICH Switzerland, 1996	[95]	General population: 8,344 subjects aged 18–60 yrs	Higher IgE among smokers than nonsmokers	
SAPIGNI Italy, 1998	[21]	General population: 1,905 subjects aged 8–73 yrs	Higher IgE among smokers than ex/nonsmokers. A dose-response relationship between IgE and cigarette consumption in males	No difference between non and exsmokers

SPT: skin-prick test; ECRHS: European Community Respiratory Health Survey.

dust or gas exposure has been described. Whether the observed association between total serum IgE and dust or gas exposure reflects a nonallergic inflammatory response or it reflects an allergic response due to exposure of specific occupational allergens is not known. However, it has previously been documented that some occupational allergens can induce serum specific IgE antibodies [18].

Finally, it is not excluded that some factors are likely to intervene directly on IgE assessment. In Norway, subjects seen in summer had higher IgE levels than those seen in other seasons of the year [17], probably due to pollen exposure during the summer in the Northern countries.

Skin prick test: relationship to sex, age, ethnic group, environmental factors

The reactivity to skin tests depends on age. There exists a hyporeactivity at birth which lasts until the age of 6 months. Later, the proportion of reactors begins to increase. The maximum of the reactivity curve has been seen among the young adults in various populations [9]. However, a decrease of the reactivity, differing according to the sex in individuals >50 yrs, was seen at the age of 14 yrs in Denmark [102]. Similarly, males and females presented different patterns, the males preceding the females by 5 yrs, in the Netherlands [40]. The higher prevalence rate of skin prick reactivity during the younger age groups can represent either a process of maturation of immunological reactivity, or a longer exposure to environmental allergens. On the contrary, the decline of skin prick reactivity in the older age groups might depend either on a real decline of immunological reactivity or on a decrease of the skin′s capacity to react to immunological solicitations. A loss of vascular bed and a reduction in histamine release were observed in the skin of older adults [9]. In general, there is no agreement on the role played by sex in skin test reactivity according to the literature. Some studies

did not find any difference [103], others did find one [102, 104], even if only in some age groups [9].

Few endogenous factors have been related to skin test reactivity. Low gestational age was identified as risk factor for sensitization to aeroallergens at school age [105]. Low birth-weight was a significant risk for skin test reactivity at the age of 2 yrs [78]. Ethnic factors also influence skin test reactivity. In a worker population, skin test reactivity was higher in non-White individuals compared to White [103]. An Australian epidemiological survey showed that skin prick test positivity was observed more in Asian immigrants and Australian-born Asians than in Australian-born non-Asian [106].

Among environmental factors related to skin test reactivity, there are irritants, allergens and residence area. Out of irritants, tobacco active smoking is presented in table 4. The role of allergenic exposure is indisputable. Skin test reactivity to pollens or house dust mites depends on the month of birth [78, 107]. Former cat ownership was significantly related to sensitization to cat dander [105]. Data collected in a study on 3,371 patients with diagnosis of allergic asthma, rhinitis, or both [36], showed that indoor allergen sensitization was strongly associated with asthma, while exclusive sensitization to pollens was associated primarily with rhinitis.

Living in urban, as compared with rural areas [40], or living in West, as compared with East Germany [108], is associated with an increased prevalence of sensitization to aeroallergens. Although, in recent years, a big effort has been made to reduce emissions from industries and automobile exhausts in the USA, Japan and some European countries, levels of air pollutants are still very high, especially in large urban areas. The prevalence of skin test reactivity resulted is statistically higher in urban *versus* rural area in the Anglo-Saxon population of the USA. In fact, a study in Japan has demonstrated that the prevalence of clinical sensitivity to cedar pollen is higher among subjects living along roads with heavy traffic but with low pollen counts in comparison with those living in cedar forests with high pollen counts but less traffic. Another study showed that the most polluted areas in the Erie county also had higher rates of eczema. Indeed, studies in animals have shown that air pollutants such as SO_2, NO_2, and O_3 increase the permeability of the mucous membrane of the airway tract, favouring the development of allergic reactions [109]. Environmental tobacco exposure is a risk factor for skin test reactivity [110]. However, no demonstration of *in utero* exposure could be given. Smoking showed an inverse effect on skin reactivity: *e.g.* SHERRILL *et al.* [57] reported that smoking tended to be less common in skin-test reactors than in nonreactors and other authors found similar results [9, 45]. WÜTHRICH *et al.* [95] reported that tobacco smoking is associated with increased IgE levels and negatively related to skin reactivity and hay fever.

Inverse associations between atopy and smoking may be explained by selection factors. Indeed, atopic subjects may be more susceptible to environmental hazards, such as the bronchial irritant effects of smoking, than the rest of the population, and, consequently, they may decide to quit or not to start smoking.

It is interesting to point out the possible interactions between smoking and atopy in affecting bronchial responsiveness. O'CONNOR *et al.* [111] observed a higher degree of bronchial responsiveness in smokers with positive skin tests, than in smokers with negative skin tests. In multivariate models, this author observed minor additional effects of IgE levels and blood eosinophils on bronchial hyperreactivity and he hypothesized a synergism of cigarette smoking and skin test reactivity in affecting bronchial hyperresponsiveness.

Additional interesting observations are present in studies obtained in subjects exposed to specific irritants in occupational settings. Higher values of specific IgE or higher prevalence of subjects with positive skin tests were reported in exposed smokers when compared to nonexposed smokers [112]. These observations suggest that smoking may facilitate sensitization in exposed subjects, increasing bronchial mucosa permeability and

Table 4. – Relationship between smoking habits and skin tests reactivity among adults

Authors	[Ref.]	Subjects	Findings	Comments
MENSINGA the Netherlands, 1990	[45]	General population: 3,258 subjects aged 14–39 yrs	Inverse association between positive skin test reactivity and cigarette smoking in males but not in females	Scratch method
NIELSEN Denmark, 1994	[102]	General population: 793 subjects aged 15–69 yrs	Higher prevalence of positive SPT in nonsmokers than in current smokers, more marked in males	Lancet. Adjusted for sex and age, exsmokers intermediate between nonsmokers and smokers for skin reactivity
MENSINGA the Netherlands, 1994	[40]	General population: 2,875 subjects aged 17–49 yrs	No association between SPT reactivity and smoking habits	Similar results in light and heavy smokers
BALDACCI Italy, 1996	[9]	General population: 2,649 subjects aged 8–75 yrs	No effect of smoking on SPT, but a trend in nonsmokers to have higher SPT positivity	
WÜTHRICH Switzerland, 1996	[95]	General population: 8,344 subjects aged 18–60 yrs	Higher prevalence of positive SPT in nonsmokers than in current smokers	Difference between former smokers and nonsmokers, more pronounced in males

SPT: skin-prick test.

thus facilitating and enhancing allergen penetration in the respiratory mucosa.

Various occupational exposures have been related to skin test reactivity [103]. Surprisingly, skin test reactivity depends also on the period of the survey. House dust mite reactivity was more frequent in subjects seen during the winter compared to the others [104]. Finally, it is well known that various medications may reduce the skin tests reactivity. Medication intake must be taken into account in the study of skin reactions.

Eosinophilia: relationship to sex, age, ethnic group, environmental factors

The eosinophil is an inflammatory cell involved in pulmonary disorders, mainly allergic. Both tissue and circulating eosinophils are involved, but only peripheral blood eosinophils have been considered in epidemiological studies. Population-based data have shown that peripheral blood eosinophilia is related to asthma prevalence and acute attacks, bronchial hyperresponsiveness, and decreased lung function level and decline [13, 43, 44]. Eosinophils are strongly related to age in childhood [113]. The relationship is unclear in adulthood [114]. Children have a higher eosinophil count than adults. The maximum of the distribution is observed at the age of 4–8 yrs. However, an epidemiological survey conducted in the Netherlands could not find any association [40]. Similarly, the relationship to sex is unclear. Eosinophilia defined as $\geqslant 275$ cells·mm^{-3} was more frequent in males than in females [45, 115]. This was not confirmed by other studies [113, 116]. Results from studies on the role of ethnic group are contradictory. Black individuals are likely to have higher eosinophil counts [115, 117], but this has not been confirmed [118, 119]. Other biological sources of variation of eosinophil count have been identified: exercise, emotional stress, physical abuse, diurnal variation beta-adrenergic agents and hormonal influence of the menstrual source [114]. However, few epidemiological evidence exists. Interpretation of results on eosinophils must consider the fact that various cut-off values have been proposed so that comparisons are not allowed. Few studies have reported on the effects of tobacco smoking on eosinophilia (table 5).

Eosinophils are also influenced by residence area and pollution. Urban residents are at higher risk of eosinophilia than rural residents [40]. Similarly, a difference in eosinophil count was seen between the

Table 5. – Relationship between smoking habits and eosinophilia

Authors	[Ref.]	Subjects	Findings	Comments
RONCHETTI Italy, 1990	[97]	179 children aged 9 yrs	Children whose parents smoke have increased absolute counts and percentages ($\geqslant$4%) of eosinophils; effect only significant in male children	Similar effect for paternal and maternal smoking. Eosinophilia increases with number of smoking parents in males, only a trend in females (small minority of mothers smoked)
MENSINGA the Netherlands, 1990	[45]	General population: 3,258 subjects aged 14–39 yrs	Current smoking related to higher eosinophil counts. Association stronger for heavy smokers ($\geqslant$15 cigarettes·day^{-1}) than light smokers	Elevated eosinophil count associated with males *versus* females. No relation of eosinophil counts between exsmokers and nonsmokers
SCHWARTZ USA, 1994	[118]	6,138 subjects age 30–74 yrs	Eosinophils positively associated with the current number of cigarettes smoked per day. No effect of pack-years and years since quitting. Increased cigarette smoking decreased percent eosinophils counts	Multiple regression models controlled for age, sex, ethnic group and body mass index. No differences in eosinophils between males and females
EL-NAWAWY Egypt, 1996	[120]	120 subjects mean aged 10 yrs	Children whose parents smoke have increased absolute counts of eosinophils, effect only significant in male children	Passive smoking increased eosinophil counts in children with frequent respiratory symptoms compared to others
JENSEN Denmark, 1998	[121]	298 smoking volunteers and 136 random controls; follow-up 6 months; mean age 44.2 yrs	No significant differences between smokers and nonsmokers for eosinophil counts and percentage	
ULRIK Denmark, 1998	[43]	665 adolescents aged 13–23 yrs	Association between daily tobacco consumption and eosinophil count approached the level of statistical significance. No effect of pack-years and eosinophil counts	
SUNYER USA, 1996	[122]	2,435 men aged >18 yrs	Eosinophil percentage positively associated with cigarettes pack·day^{-1} smoked	AIDS cohort study, homosexual/bisexual volunteer men (AIDS-free) from four US sites

AIDS: acquired immunodeficiency syndrome.

subjects living in an area polluted by sulphur dioxide and fluoride than in an unpolluted area in Norway. Furthermore, a recent exposure was related to a higher eosinophilia [123].

Relationship among skin reactivity, IgE, eosinophilia and respiratory health outcomes

Skin test reactivity has been used to categorize individuals as atopic or nonatopic. However, eosinophilia and elevated serum IgE levels, which correlate with skin test reactivity, have also been used in many studies as alternative methods to characterize atopic subjects. These three main phenotypic markers are commonly utilized to identify the "atopic" state.

Indeed, different categories of respiratory symptoms and conditions may be associated with different phenotypic markers. For instance, BURROWS *et al.* [4] have reported that asthma, in a random sample of residents in Tucson, Arizona, was strongly correlated with age- and sex-adjusted serum IgE level, independently of skin test reactivity; on the contrary, hay fever was closely associated with skin test reactivity to a set of common aeroallergens.

In another population-based study in The Netherlands, MENSINGA *et al.* [45] analysed the relationship of eosinophilia and skin test reactivity to respiratory symptoms. They reported an association of eosinophilia with chronic cough, bronchitis, and dyspnoea, while skin test positivity and eosinophilia together were associated with persistent wheeze and asthma attacks. TOLLERUD *et al.* [12], in the Normative Aging Study, found a significant association of skin test positivity with hay fever, but not with symptoms of wheeze or cough and phlegm. Eosinophilia was associated with asthma and with phlegm production, and serum total IgE concentration was more strongly associated with asthma. In the second survey on the Italian general population study of Po river Delta [124], IgE and skin prick reactivity resulted associated with asthma and rhinitis. However, IgE appeared more closely associated with asthma while skin prick reactivity showed a moderately stronger association with rhinitis. Furthermore, cough was also significantly associated with skin reactivity, but only in females [9]. In the same general population the relationship between skin reactivity and total IgE level was found to vary with the specific categories of allergens: it was significant for house-dust mites and pollens, but not for moulds and animal danders. Therefore, both biomarkers of atopy should be used in epidemiological studies [21].

STEIN *et al.* [125] demonstrated that methacholine responsiveness, peak flow variability and markers of atopy (total IgE and skin test reactivity) identify three different wheezing phenotypes in childhood: "transient early wheezing" limited to the first three years of life and unrelated to increased airway lability; "nonatopic wheezing" of the infancy and early school years associated with positive peak flow variability but not with hyperresponsiveness; and "IgE-associated wheeze/asthma" associated with persistent wheezing at any age and with methacholine hyperresponsiveness, peak flow variability, and markers of atopy.

Also the Dutch ECRHS Group [126] confirmed the close relationship of skin test positivity with reported symptoms of nasal allergy in a general population. In particular, for each tested allergen, a skin test positivity together with a positive specific IgE measurement were the best predictors of the corresponding nasal symptoms. High levels of total IgE may be considered an indicator of greater dysregulation of the immune system in atopic allergy. Eosinophil count may be considered an aspecific indicator of nasal allergy as it was associated with all nasal symptom groups: symptoms due to exposure to indoor allergens only, symptoms due to exposure to outdoor allergens only, and symptoms due to exposure to both indoor and outdoor allergens.

In a Spanish study [127], the two markers of atopy, total IgE and skin test reactivity, were found differently related to bronchial asthma according to the different stages of life: skin test reactivity was more useful as a predictive factor in children, while IgE determination was more useful in adults.

Moreover, blood eosinophilia at 3 months of age has been found to correlate significantly to cord blood IgE levels and to skin prick test reactivity later on during a follow-up period of 18 months. Furthermore, the association between increased blood eosinophil counts and a subsequent diagnosis of atopic disease suggests the main predictive role of this atopic marker [41]. On the contrary, an increased basophil count at birth was not a good predictive marker of atopy [128]. In addition, TERHO *et al.* [10], investigating the predictive value of skin reactivity, smoking, and living in a farming environment in the development of chronic bronchitis, found that atopy and smoking seemed to have an additive effect on both the prevalence and incidence of chronic bronchitis, thus supporting the so-called "Dutch hypothesis" about the natural history of chronic bronchitis [129].

Many studies, exploring the natural history of asthma and its relation to allergic responses, have examined the relation between airway hyperresponsiveness and atopy. PEAT *et al.* [130] reported that young adults who are sensitized to common allergens have a high chance to get airway hyperresponsiveness, even in the absence of symptoms; this is further evidence of the fundamental role of IgE-mediated responses in the natural history of airway hyperresponsiveness throughout childhood and adulthood. Phenotypes of asthma described by IgE, SPT and eosinophilia in children and adults have been reported [131] and discussed with regard to criteria applied in studies and their limitations [132]. Figure 3 depicts the number of adults and children with atopy according to the three classic definitions: any skin wheal $\geqslant 3$ mm, total IgE level $\geqslant 100\,kU \cdot L^{-1}$ and blood eosinophilia >5%. It showed that 8.5% of asthmatic adults and 1.5% of asthmatic children were not atopic by any criterion and that there was some heterogeneity, particularly in adults.

In several cross-sectional studies, total serum IgE has been related to reduced pulmonary function, a marker of obstructive lung disease [45, 65, 133–137]. In recent longitudinal studies, elevated total serum IgE has also been related to an increased rate of

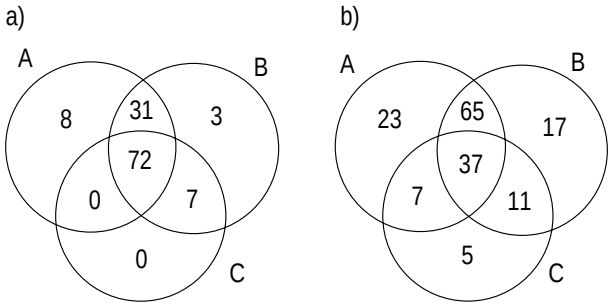


Fig. 3. – Comparison of asthma phenotypes in: a) 135 children, (2 subjects with missing data) and b) 213 adults (18 subjects with missing data), of the Epidemiological Study of the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA). The following intermediate phenotypes are described in asthmatic probands of the EGEA study: A: skin prick testing (SPT) ($\geqslant$3 mm); B: total serum IgE (IgE) $\geqslant$100 IU·mL^{-1}; and C: blood eosinophilia (Eosinophils) >5% for 30 adults and 12 children, at least on the data was missing. Adapted from reference [132].

decline in pulmonary function of nonsmokers and exsmokers [135] as well as smokers [138]. Thus, elevated total serum IgE has been suggested as a possible risk factor for developing obstructive lung disease as well as being significantly associated with the asthmatic phenotype [136].

Why is there a relationship between total serum IgE and airways obstruction? Increased permeability of the airways to less frequently encountered allergens could be one explanation [139]. In smokers, another explanation may be the effects of smoking on leukocytes in the lung and the peripheral blood including the lymphocytes and "dysregulation" of cytokines [90, 140]. IgE appears to be a biomarker of changed or impaired lymphocyte-cytokine activity which has a significant contribution in causing obstructive lung disease, and especially chronic obstructive lung disease [140]. In nonsmokers and especially in those without atopy one or more other unrecognized factors may be relevant for obstructive lung disease.

The lack of a relationship by specific allergy and pulmonary function has been reported [46, 133, 141]. However, all these investigators have used a grouped variable including one or more specific allergens and may thus have obscured any significant relationship between specific allergy and reduced lung function level. In children with atopy to HDM or cat, however, there has been observed an impaired growth in lung function, as assessed by spirometry, from 9–15 yrs [142].

In adults from a general population there was observed a dose-response relationship between HDM antibody levels and reduced lung function indicating a causal relationship [143]. House dust mite allergy in selected groups of adults has been associated with asthmatic phenotype [144]. Even among elderly, allergic sensitization (atopy) has been associated with a more rapid decline in FEV_1 than in those without atopy [140]. A clinical study suggests that moving to "healthy homes" with a reduction in house dust mite exposure give improvements in FEV_1, in medicine score, in symptom score and in total serum IgE [145].

Some [146, 147], but not all studies [143] in general populations of adults have observed a significant association between total serum IgE and nonspecific bronchial responsiveness after adjusting for confounders. In a survey limited to subjects with obstructive lung disease there was also an association between increasing total serum IgE levels and bronchial responsiveness, while not with increasing number of serum specific IgE antibodies [148]. Furthermore, a longitudinal study of children [151] observed that the overall tendency to bronchial responsiveness and the tendency to retain bronchial responsiveness were closely related to total serum IgE.

In children, there has been found a relationship between allergic sensitization (atopy) and nonspecific bronchial responsiveness in cross-sectional and longitudinal studies [93, 149–151]. This has been suggested to include primarily indoor allergens. Atopy may reflect a tendency to overproduce mediators in response to allergen-exposure, while nonspecific bronchial responsiveness may reflect the propensity of the target cells in the bronchi to respond to allergy-related mediators such as histamine [152].

Recent reports have observed a relationship between serum specific IgE antibodies and bronchial responsiveness in young adults, but it may be limited to some allergens at least in adults [143, 146, 147, 153, 154].

Conclusion

Epidemiological evidence confirms that different groups of respiratory symptoms and conditions may be associated with distinct phenotypic markers.

In fact, many pertinent studies have demonstrated a closer relationship of total IgE to asthma and a stronger association between skin reactivity and rhinitis [4, 12, 124], but there are also other findings that suggest a relationship of atopic predisposition, as assessed by prick test reactions or eosinophilia, to other respiratory problems, in particular, those chronic bronchitis related, supporting the "Dutch hypothesis" [45, 129]. Thus, future investigations will be devoted to developing a more precise and consistent definition of atopy which is a prerequisite for elucidating the complex relationship among atopy, respiratory disease susceptibility and respiratory health outcome.

Furthermore, longitudinal population studies are required to amplify the cross sectional observations, in fact they will be more and more important in order to determine in which period of life the onset of the atopic status is prognostically relevant. These studies will also allow evaluation of the relationship of the age of onset and the severity of atopy with the persistence and the severity of respiratory symptoms. As exposure and sensitization to certain allergens have shown a close relation with clinical diagnoses, there is an urgent need to assess procedures of allergen avoidance and of reducing allergens in the environment to determine the effect on patient symptoms, exacerbation rate, use of medication and health costs evaluation.

References

1. Rapaport HG. Clemens von Pirquet and allergy. *Ann Allergy* 1973; 31: 467–75.
2. Van Arsdel PP, Larson EB. Diagnostic tests for patients with suspected allergic disease. *Ann Intern Med* 1989; 110: 304–312.
3. Coca AF, Cooke RA. On the classification of the phenomena of hypersensitiveness. A study of atopic reagines. *J Immunol* 1923; 8: 163–171.
4. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989; 320: 271–277.
5. Bousquet J, Lockey R, Malling H-J. WHO Position Paper. Allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998; 53: (Suppl.), 5–42.
6. Sunyer J, Anto J, Kogevinas M, *et al.* Risk factors for asthma in young adults. *Eur Respir J* 1997; 10: 2490–2494.
7. Hulka B, Margolin B. Methodological issues in epidemiologic studies using biologic markers. *Am J Epidemiol* 1992; 135: 200–209.
8. Merrett T. Quantification of IgE both as total immunoglobulin and as allergen-specific antibody. *In*: Kay A, ed. Allergy and allergic diseases. Oxford, Blackwell Science, 1997; pp. 1012–1034.
9. Baldacci S, Modena P, Carrozzi L, *et al.* Skin prick test reactivity to common aeroallergens in relation to total IgE, respiratory symptoms, and smoking in a general population sample of northern Italy. *Allergy* 1996; 51: 149–156.
10. Terho EO, Koskenvuo M, Kaprio J. Atopy: a predisposing factor for chronic bronchitis in Finland. *J Epidemiol Community Health* 1995; 49: 296–298.
11. Weller PF, Lim K, Wan HC, *et al.* Role of the eosinophil in allergic reactions. *Eur Respir J* 1996; 9: 109s–115s.
12. Tollerud DJ, O'Connor GT, Sparrow D, Weiss ST. Asthma, hay fever and phlegm production associated with distinct patterns of allergy skin test reactivity, eosinophilia, and serum IgE levels. *Am Rev Respir Dis* 1991; 144: 776–781.
13. Jansen DF, Rijcken B, Schouten JP, *et al.* The relationship of skin test positivity, high serum total IgE levels, and peripheral blood eosinophilia to symptomatic and asymptomatic airway hyperresponsiveness. *Am J Respir Crit Care Med* 1999; 159: 924–931.
14. Pepys J. Atopy. *In*: Gill P, Coombs R, Lachmann P, eds. Clinical Immunology. 3rd edn. Oxford, Blackwell Science, 1975; pp. 877–902.
15. Jarvis D, Burney P. Epidemiology of atopy and atopic disease. In: Kay A, ed. Allergy and allergic diseases. Oxford, Blackwell Science, 1997; pp. 1208–1224.
16. Burney P, Malmberg E, Chinn S, Jarvis D, Luczynska C, Lai E. The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 1997; 99: 314–322.
17. Omenaas E, Bakke P, Elsayed S, Hanoa R, Gulsvik A. Total and specific serum IgE levels in adults: relationship to sex, age and environmental factors. *Clin Exp Allergy* 1994; 24: 530–539.
18. Zetterström O, Osterman K, Machado L, Johansson S. Another smoking hazard: raised serum IgE concentration and increased risk of occupational allergy. *BMJ* 1981; 283: 1215–1217.
19. Burr M, Leger A, Bevan C, Merrett T. A community survey of asthmatic characteristics. *Thorax* 1975; 30: 663–668.
20. Cline M, Burrows B. Distribution of allergy in a population sample residing in Tucson, Arizona. *Thorax* 1989; 44: 425–431.
21. Sapigni T, Biavati P, Simoni M, *et al.* The Po River Delta Respiratory Epidemiological Survey: an analysis of factors related to level of total serum IgE. *Eur Respir J* 1998; 11: 278–283.
22. Mensinga T, Scouthen J, Weiss S, Kauffmann H, Speizer F, Van der Lende R. The relationship between allergy, respiratory symptoms, and pulmonary function. *In*: Sluiter H, Van der Lende R, eds. Bronchitis IV. Assen, Royal van Gorcum, 1989; pp. 35–41.
23. Gassner M. Immunological-allergological reactions with changed environmental conditions. *Schweiz Rundsch Med Prax* 1992; 81: 426–430.
24. Nakagomi T, Itaya H, Tominage T, Yamaki M, Hisamatsu S, Nakagomi O. Is atopy increasing?. *Lancet* 1994; 343: 121–112.
25. Colloff MJ, Ayres J, Carswell F, *et al.* The control of allergens of dust mites and domestic pets: a position paper. *Clin Exp Allergy* 1992; 22: (Suppl.), 1–28.
26. Van Metre TE, Adkinson NF, Kagey-Sobotka A, Marsh DG, Norman PS, Rosenberg GL. How should we use skin testing to quantify IgE sensitivity? *J Allergy Clin Immunol* 1990; 86: 583–627.
27. Peat JK, Woolcock AJ. Sensitivity to common allergens: relation to respiratory symptoms and bronchial hyperresponsiveness in children from three different climatic areas of Australia. *Clin Exp Allergy* 1991; 21: 573–581.
28. Baldacci S, Carrozzi L, Viegi G, Giuntini C. Assessment of respiratory effect of air pollution: study design on general population samples. *JEPTO* 1997; 16: 77–83.
29. Haahtela T, Björksten F, Heiskala M, Suoniemi I. Skin prick test reactivity to common allergens in Finnish adolescents. *Allergy* 1980; 35: 425–431.
30. Barbee RA, Lebowitz MD, Thompson HC, Burrows B. Immediate skin-test reactivity in a general population sample. *Ann Intern Med* 1976; 84: 129–133.
31. Sin A, Köse S, Terzioglu E, Kokuludag A, Sebik F, Kabakçi T. Prevalence of atopy in young healthy population, in Izmir, Turkey. *Allergol Immunopathol* 1997; 25: 80–84.
32. Nowak D, Heinrich J, Jörres R, *et al.* Prevalence of respiratory symptoms, bronchial hyperresponsiveness and atopy among adults: west and east Germany. *Eur Respir J* 1996; 9: 2541–2552.
33. Herxheimer H, McInroy P, Sutton KH. The evaluation of skin tests in respiratory allergy. *Acta Allergol* 1954; 7: 380–396.
34. Freidhoff LR, Meyers DA, Bias WB, Chase GA, Hussain R, Marsh DG. A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. I. Epidemiology of reported allergy and skin test positivity. *Am J Med Genet* 1981; 9: 323–340.
35. Sibbald B, Rink E. Epidemiology of seasonal and perennial rhinitis: clinical presentation and medical history. *Thorax* 1991; 46: 895–901.
36. Boulet LP, Turcotte H, Laprise C, *et al.* Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. *Clin Exp Allergy* 1997; 27: 52–59.
37. Dreborg S. Standardization of allergenic preparations

by *in vitro* and *in vivo* methods. *Allergy* 1993; 48: 63–70.
38. Viegi G, Diviggiano E, Carrozzi L, Vellutini M, Prediletto R. Distribuzione della cutireattività agli allergeni respiratori in un sottocampione della popolazione generale. *Lotta contro la TBC e malattie polmonari soc* 1987; 3: 573–576.
39. Venge P. The eosinophil. *In*: Godard P, Bousquet J, Michel FB, eds. Advances in allergology and clinical immunology. ed. Parthenon Publishing Group, 1992; pp. 175–185.
40. Mensinga TT, Schouten JP, Rijcken B, Weiss ST, Van der lende R. Host factors and environmental determinants associated with skin test reactivity and eosinophilia in a community-based population study. *Ann Epidemiol* 1994; 4: 382–392.
41. Borres MP, Odelram H, Irander K, Kjellman NI, Björksten B. Peripheral blood eosinophilia in infants at 3 months of age is associated with subsequent development of atopic disease in early childhood. *J Allergy Clin Immunol* 1995; 95: 694–698.
42. Kajosaari M, Saarinen UM. Evaluation of laboratory tests in childhood allergy. Total serum IgE, blood eosinophilia and eosinophil and mast cells in nasal mucosa of 178 children aged 3 years. *Allergy* 1981; 36: 329–335.
43. Ulrik CS. Eosinophil and pulmonary function: An epidemiologic study of adolescents and young adults. *Ann Allergy Asthma Immunol* 1998; 80: 487–493.
44. Mensinga TT, Schouten JP, Weiss ST, van der Lende R. Relationship of skin test reactivity and eosinophilia to level of pulmonary function in a community-based population study. *Am Rev Respir Dis* 1992; 146: 638–643.
45. Mensinga T, Schouten J, Rijcken B, Weiss S, Speizer F, Van der Lende R. The relationship of eosinphilia and positive skin test reactivity to respiratory symptom prevalence in a community-based study. *J Allergy Clin Immunol* 1990; 86: 99–107.
46. Taylor R, Gross E, Joyce H, Holland F, Pride N. Smoking, allergy, and the differential white blood cell count. *Thorax* 1985; 40: 17–22.
47. Kauffmann D, Annesi I, Oryszczyn MP. The relationship between smoking and allergy. *In*: Sluiter HJ, Van der Lende R, eds. Bronchitis IV. Assen, Van Gorcum, 1989; pp. 57–70.
48. Taylor KL, Luksza AR. Peripheral blood eosinophil counts and bronchial responsiveness. *Thorax* 1987; 42: 542–556.
49. Meyers D, Postma D, Panhuysen C, *et al.* Evidence for regulating total serum IgE levels mapping to chromosome 5. *Genomics* 1994; 23: 464–470.
50. Cookson W, Sharp P, Lynch J, Faux A, Hopkin J. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. *Lancet* 1989; i: 1292–1295.
51. Hanson B, Kronenburg R, Johnson B, Blumenthal M. Pulmonary function, serum IgE levels and specific IgE immune responses in monozygotic twins reared apart. *J Allergy Clin Immunol* 1985; 75: 155.
52. Hopkins J. The genetics of asthma. *Arch Dis Child* 1993; 68: 721–723.
53. The European Community Respiratory Health Survey Group. Genes for asthma? An analysis of the European Community Respiratory Health Survey. *Am J Respir Crit Care Med* 1997; 156: 1773–1780.
54. Marone G, Poto S, Clesino D, Bonini S. Human basophil releasability. III. Genetic control of human basophil releasability. *J Immunol* 1986; 137: 3588–3592.
55. Magnusson CGM. Maternal smoking influence cord serum IgE and IgD levels increase the risk for subsequent infant allergy. *J Allergy Clin Immunol* 1986; 78: 898–904.
56. Johnson CC, Peterson EL, Ownby DR. Gender differences in total and allergen-specific Immunoglobulin E (IgE) concentrations in a population-based cohort from birth to age four years. *Am J Epidemiol* 1998; 147: 1145–1152.
57. Sherrill D, Halonen M, Burrows B. Relationships between total serum IgE, atopy, and smoking: a twenty-year follow-up analysis. *J Allergy Clin Immunol* 1994; 94: 954–962.
58. Merrett T, Burr M, St Leger A, Merrett J. Circulating IgE levels in the over-seventies. *Clin Allergy* 1980; 10: 433–439.
59. Haahtela T, Jaakonmäki I. Relationship of allergen-specific IgE antibodies, skin prick tests and allergic disorders in unselected adolesents. *Allergy* 1981; 36: 251–256.
60. Gergen P, Turkeltaub P, Kovar M. The prevalence of allergic skin test reactivity to eight common aeroallergens in the U.S. population: results from the second National Health and Nutrition Examination Survey. *J Allergy Clin Immunol* 1987; 80: 669–679.
61. Barbee R, Halonen M, Lebowitz M, Burrows B. Distribution of IgE in a community population sample: correlations with age, sex, and allergen skin test reactivity. *J Allergy Clin Immunol* 1981; 68: 106–111.
62. Jarvis D, Luczynska C, Chinn S, Burney P. The association of age, gender and smoking with total IgE and specific IgE. *Clin Exp Allergy* 1995; 25: 1083–1091.
63. Petridou E, Kanariou M, Liatsis M, *et al.* Factors influencing serum immunoglobulin E levels in Greek children. *Allergy* 1995; 50: 210–214.
64. Oryszczyn M, Annesi I, Neukirch F, Dore M, Kauffmann F. Relationship of total IgE level, skin prick test response, and smoking habits. *Ann Allergy* 1991; 67: 355–359.
65. Jensen E, Pedersen B, Schmidt E, Dahl R. Serum IgE in nonatopic smokers, nonsmokers, and recent exsmokers: Relation to lung function, airway symptoms, and atopic predisposition. *J Allergy Clin Immunol* 1992; 90: 224–229.
66. Grundbacher FJ, Massie FS. Levels of immunoglobulin G, M, A and E at various ages in allergic and non allergic black and white individuals. *J Allergy Clin Immunol* 1985; 75: 651–658.
67. Haus M, Heese HD, Weinberg EG, Potter PC, Hall JM, Malherbe D. The influence of ethnicity, an atopic family history, and maternal ascariasis on cord blood serum IgE concentrations. *J Allergy Clin Immunol* 1988; 82: 179–189.
68. Oryszczyn MP, Annesi-Maesano I, Campagna D, Sahuquillo J, Huel G, Kauffmann F. Head circumference at birth and maternal factors related to cord blood IgE. *Clin Exp Allergy* 1999; 29: 334–341.
69. Godfrey KM, Barker DJP, Osmond C. Disporportionate fetal growth and raised IgE concentration in adult life. *Clin Exp Allergy* 1994; 24: 641–648.
70. Vellutini M, Viegi G, Parrini D, *et al.* Serum immunoglobulins E are related to menstrual cycle. *Eur J Epidemiol* 1997; 13: 931–935.

71. Pope A, Patterson R, Burge H. Indoor allergens. Assessing and controlling adverse health effects Washington D.C., National Academy Press, 1993.
72. Platts-Mills T, Sporik R, Ward G, *et al.* Dose-response relationships between asthma and exposure to indoor allergens. *Progr Allergy Clin Immunol* 1995; 3: 90–96.
73. Sporik R, Holgate S, Platts-Mills T, Cogswell J. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. *N Engl J Med* 1990; 323: 502–507.
74. Luczynska C, Li Y, Chapman M, Platts-Mills T. Airborne concentratuions and particle size distribution of allergen derived from domestic cats (Felis domesticus): Measurements using cascade impactor, liquid impinger and two site monoclonal antibody assay Fel dI. *Am Rev Respir Dis* 1990; 141: 361–367.
75. Seaton A, Gooden D, Brown K. Increase in asthma: a more toxic environment or a more susceptible population. *Thorax* 1994; 49: 36–41.
76. Peat J, van den Berg R, Green W, Mellis CM, Leeder SR, Woolcock AJ. Changing prevalence of asthma in Australian children. *BMJ* 1994; 308: 1591–1596.
77. Harving H, Korsgaard J, Dahl R. House dust mites and associated environmental conditions in Danish homes. *Allergy* 1993; 48: 106–109.
78. Rugtveit J. Environmental factors in the first month of life and the possible relationship to later development of hypersensitivity. *Allergy* 1990; 45: 154–156.
79. Burr M, Limb E, Maguire M, *et al.* Infant feeding, wheezing and allergy: a prospective study. *Arch Dis Child* 1993; 68: 724–728.
80. Bjerke T, Hedegaard M, Henriksen TB, Nielsen BW, Schitz PO. Several genetic and environmental factors influence cord blood IgE concentration. *Pediatr Allergy Immunol* 1995; 5: 88–94.
81. Berciano FA, Crespo M, Bao CG, Alvarez FV. Serum levels of total IgE in non-allergic children. Influence of genetic and environmental factors. *Allergy* 1987; 42: 276–283.
82. Von Mutius E, Martinez F, Fritzsch C, Nicolai T, Reitmeir P, Thiemann H. Skin test reactivity and number of siblings. *BMJ* 1994; 308: 692–695.
83. Strachan D, Griffiths J, Anderson H, Johnston I. Allergic sensitisation and position in the sibship: a national study of young British adults. *Thorax* 1994; 49: 1053P.
84. Wjst M, Heinrich J, Liu P, *et al.* Indoor factors and IgE levels in children. *Allergy* 1994; 49: 766–771.
85. Shirakawa T, Enomoto T, Shimazu S, Hopkin J. The inverse association between tuberculin responses and atopic disorder. *Science* 1997; 275: 77–79.
86. Shaheen S, Aaby P, Hall A, *et al.* Measles and atopy in Guinea-Bissau. *Lancet* 1996; 347: 1792–1796.
87. Burrows B, Halonen M, Barbee R, Lebowitz M. The relationship of serum immunoglobulin E to cigarette smoking. *Am Rev Respir Dis* 1981; 124: 523–525.
88. Romagnani S, Ricci M. Present views on the regulation of human IgE synthesis. *ACI News* 1990; 2: 192–196.
89. Bloom J, Halonen M, Dunn A, Pinnas J, Burrows B. Pneumococcus-specific immunoglobulin E in cigarettes smokers. *Clin Allergy* 1986; 16: 25–32.
90. Holt P. Immune and inflammatory function in cigarette smokers. *Thorax* 1987; 42: 241–249.
91. Osterman K, Zetterström O, Johansson S. Coffee worker's allergy. *Allergy* 1982; 37: 313–322.
92. Sunyer J, Anto J, Sabria J, *et al.* Risk factors of soybean epidemic asthma. *Am Rev Respir Dis* 1992; 145: 1098–1102.
93. Backer V, Ulrik CS, Wendelboe D, *et al.* Distribution of serum IgE in children and adolescents aged 7 to 16 years in Copenhagen, in relation to factors of importance. *Allergy* 1992; 47: 484–489.
94. Lai CKW, Ho SC, Lau J, *et al.* Respiratory symptoms in elderly chinese living in Hong Kong. *Eur Respir J* 1995; 8: 2055–2061.
95. Wüthrich B, Schindler C, Medici TC, Zellweger JP, Leuenberger PH , the SAPALDIA team. IgE levels, atopy markers and hay fever in relation to age, sex and smoking status in a normal adult swiss population. *Int Arch Allergy Immunol* 1996; 111: 396–402.
96. Michel F, Bousquet J, Greillier P, Robinet-Levy M, Couloumb Y. Comparison of cord blood IgE concentrations and maternal allergy for the prediction of atopic diseases in infancy. *J Allergy Clin Immunol* 1980; 65: 422–430.
97. Ronchetti R, Macri F, Ciofetta G, *et al.* Increased serum IgE and increased prevalence of eosinophilia in 9 year old children of smoking parents. *J Allergy Clin Immunol* 1990; 86: 400–407.
98. Arshad S, Stevens M, Hide D. The effect of genetic and environmental factors on the prevalence of allergic disorders at the age of two years. *Clin Exp Allergy* 1993; 23: 504–511.
99. Martinez F, Antognoni G, Macri F, *et al.* Parenteral smoking enhances bronchial responsiveness in nine-year-old children. *Am Rev Respir Dis* 1988; 138: 518–523.
100. Strachan DP, Cook DG. Health effects of passive smoking. 5. Parental smoking and allergic sensitization in children. *Thorax* 1998; 53: 117–123.
101. Oryszczyn M-P, Annesi I, Neukirch F, Doré M-F, Kauffmann F. Longitudinal observations of serum IgE and skin prick test response. *Am J Respir Crit Care Med* 1995; 151: 663–668.
102. Nielsen NH, Svendsen UG, Madsen F, Dirksen A. Allergen skin test reactivity in an unselected Danish population. *Allergy* 1994; 49: 86–91.
103. Chan-Yeung MB, Vedal S, Lam S, Enarson D. Immediate skin reactivity and its relation to age, sex, smoking and occupational exposure. *Arch Env Health* 1985; 40: 53–57.
104. Plaschke P, Janson C, Norman E, *et al.* Skin prick tests and specific IgE in adults from three different areas of Sweden. *Allergy* 1996; 51: 461–472.
105. Kuehr J, Frischer T, Karmaus W, *et al.* Early childhood risk factors for sensitization at school age. *J Allergy Clin Immunol* 1992; 90: 358–363.
106. Leung RC, Carlin JB, Burdon JG, Czarny D. Asthma, allergy and atopy in Asian immigrants in Melbourne. *Med J Aust* 1994; 161: 418–425.
107. Karachaliou F-H, Panagiotopoulou K, Manousakis M, Sinaniotis K, Papageorgiou F. Month of birth, atopic disease, and sensitization to common aeroallergens in Greece. *Pediatr Allergy Immunol* 1995; 6: 216–219.
108. Von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Roell G, Thiemann HH. Prevalence of asthma and atopy in two areas of west and east Germany. *Am J Respir Crit Care Med* 1994; 149: 358–364.
109. Blumenthal M, Blumenthal M, Bousquet J, *et al.* Evidence for an increase in atopic disease and possible causes. *Clin Exp Allergy* 1993; 23: 484–492.
110. Weiss ST, Tager IB, Munoz A, Speizer FE. The

relationship of respiratory infections in early childhood to the occurrence of increased levels of bronchial responsiveness and atopy. *Am Rev Respir Dis* 1985; 131: 573–578.
111. O'Connor GT, Sparrow D, Segal MR, Weiss ST. Smoking, atopy and methacholine airway responsiveness among middle-aged and elderly men. *Am Rev Respir Dis* 1989; 140: 1520–1526.
112. Venables KM, Dally MB, Nunn AJ, *et al.* Smoking and occupational allergy in workers in a platinum refinery. *BMJ* 1989; 299: 939–942.
113. Taylor MR, Holland CV, Spencer R, Jackson JF, O'Connor GI, O'Donnell JR. Haematological reference ranges for schoolchildren. *Clin Lab Haematol* 1997; 19: 1–15.
114. Nutman TB, Ottesen E, Cohen SG. Eosinophilia and eosinophil-related disorders. *In*: Middleton E Jr, Reed C, Ellis EF, Adkinson NF Jr, Yunginger JW, eds. Allergy, Principles and Practice. St Louis, Washington, 1988; pp. 861–887.
115. Bain B, Seed M, Gosland I. Normal values for peripheral blood white cell counts in women of four different ethnic origins. *J Clin Pathol* 1984; 37: 188–193.
116. Ulrik CS. Peripheral eosinophil counts as a marker of disease activity in intrinsic and extrinsic asthma. *Clin Exp Allergy* 1995; 25: 820–827.
117. Nduka N, Aneke C, Maxwell-Owhochuku S. Comparison of some haematological indices of africans and Caucasians resident in the same Nigerian environment. *Haematologia* 1988; 21: 57–63.
118. Schwartz J, Weiss ST. Cigarette smoking and peripheral blood leucocyte differentials. *Ann Epidemiol* 1994; 4: 236–242.
119. Tollerud DJ, Weiss ST, Brown LM, Malonev EM, Blattner ?. Racial differences in serum IgE level and eosinophil count in healthy smokers and non smokers. *Am Rev Respir Dis* 1992; 145: A538.
120. El-Nawawy A, Soliman AT, El-Azzouni O, El-Sayed A, Demian S, El-Sayed M. Effect of passive smoking on frequency of respiratory illnesses and serum immunoglobulin-E (IgE) and interleukine-4 (IL-4) concentrations in exposed children. *J Trop Pediatr* 1996; 42: 166–169.
121. Jensen EJ, Pedersen B, Narvestadt E, Dahl R. Blood eosinophil and monocyte counts are related to smoking and lung function. *Respir Med* 1998; 92: 63–69.
122. Sunyer J, Munoz A, Peng Y, *et al.* Longitudinal relation between smoking and white blood cells. *Am J Epidemiol* 1996; 144: 734–741.
123. Wieslander G, Norbäck D, Björnsson E, Janson C, Boman G. Asthma and the indoor environment: the significance of emission of formaldehyde and volatile organic compounds from newly painted indoor surfaces. *Int Arch Occup Environment Health* 1997; 69: 115–124.
124. Baldacci S, Viegi G, Paoletti P, *et al.* Skin prick tests reactivity to common airborne allergens and associated conditions in a general population sample of North Italy. *Eur Respir J* 1993; 6: 624s.
125. Stein RT, Holberg CJ, Morgan WJ, *et al.* Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. *Thorax* 1997; 52: 946–952.
126. Droste JH, Kerhof M, de Monchy JG, Schouten JP, Rijcken B. Association of skin test reactivity, specific IgE, total IgE, and eosinophils with nasal symptoms in a community-based population study. The Dutch ECRHS Group. *J Allergy Clin Immunol* 1996; 97: 922–932.
127. Pereira Vega A, Sanchez Ramos JL, Maldonado Perez JA, Ayerbe Garcia R, Gomez Entrena M, Gravalos Guzman J. Relation between asthma and atopy markers in children and young adults. *Arch Bronconeumol* 1997; 33: 272–277.
128. Calbi M, Giacchetti L, Coppola A, Triggiani M. Basophil count in neonates is not suitable for atopy predictivity. *J Investig Allergol Clin Immunol* 1996; 6: 383–387.
129. Sluiter HJ, Koëter GH, de Monchy JGR, Postma DS, de Vries K, Orie NGM. The Dutch Hypothesis (chronic non-specific lung disease) revisited. *Eur Respir J* 1991; 4: 479–489.
130. Peat JK, Toelle BG, Dermand J, van den Berg R, Britton WJ, Woolcock AJ. Serum IgE levels, atopy, and asthma in young adults: results from a longitudinal cohort study. *Allergy* 1996; 51: 804–810.
131. Kauffmann F, Dizzier M-H, Pin I, *et al.* Epidemiological study of the genetics and environment of asthma, bronchial hyperresponsiveness, and atopy. *Am J Respir Crit Care Med* 1997; 156: S123–S129.
132. Wiesch D, Meyers D, Samet J, Bleecker E. Classification of the asthma phenotype in genetic studies. In: Liggett S, Meyers D, eds. The genetics of asthma. New York, Marcel Dekker, 1996; pp. 421–442.
133. Burrows B, Knudson R, Cline M, Lebowitz M. A reexamination of risk factors for ventilatory impairment. *Am Rev Respir Dis* 1988; 138: 829–836.
134. Parker D, O'Connor G, Sparrow D, Segal M, Weiss S. The relationship of nonspecific airway responsiveness and atopy to the rate of decline of lung function. *Am Rev Respir Dis* 1990; 141: 589–594.
135. Annesi I, Oryszczyn M, Frette C, Neukirch F, Orvoen-Frija E, Kauffmann F. Total circulating IgE and FEV1 in adult men. An epidemiological longitudinal study. *Chest* 1992; 101: 642–648.
136. Dow L, Coggon D, Campell M, Osmond C, Holgate S. The interaction between immunoglobulin E and smoking in airflow obstruction in the elderly. *Am Rev Respir Dis* 1992; 146: 402–407.
137. Omenaas E, Bakke P, Eide G, Elsayed S, Gulsvik A. Total serum IgE and FEV1 by respiratory symptoms and obstructive lung disease in adults of a Norwegian community. *Clin Exp Allergy* 1995; 25: 682–689.
138. Tracey M, Villar A, Dow L, Coggon D, Lampe F, Holgate S. The influence of increased bronchial responsiveness, atopy, and serum IgE on decline in FEV1. *Am J Respir Crit Care Med* 1995; 151: 656–662.
139. Chan-Yeung M, Abboud R, Dy Buncio A, Vedal S. Peripheral leucocyte count and longitudinal decline in lung function. *Thorax* 1988; 43: 462–466.
140. Dow L. Desperately seeking a solution - total serum immunoglobulin E and airways obstruction. *Clin Exp Allergy* 1995; 25: 673–677.
141. Annesi I, Oryszczyn M, Neukirch F, Orvoen-Frija E, Korobaeff M, Kauffmann F. Relationship of upper airways disorders to FEV1 and bronchial hyperresponsiveness in an epidemiological study. *Eur Respir J* 1992; 5: 1104–1110.
142. Sherrill D, Sears M, Lebowitz M, *et al.* The effects of airway hyperresponsiveness, wheezing, and atopy on longitudinal pulmonary function in children: a 6-year follow-up study. *Pediatr Pulmonol* 1992; 13: 78–85.
143. Omenaas E, Bakke P, Eide G, Elsayed S, Gulsvik A.

Serum house dust mite antibodies: predictor of increased bronchial responsiveness in adults of a community. *Eur Respir J* 1996; 9: 919–925.
144. Björnsson E, Norbäck D, Janson C, *et al.* Asthmatic symptoms and indoor levels of micro-organisms and house dust mites. *Clin Exp Allergy* 1995; 25: 423–431.
145. Harving H, Korsgaard J, Dahl R. Clinical efficacy of reduction in house-dust mite exposure in specially designed, mechanically ventilated healthy homes. *Allergy* 1994; 49: 866–870.
146. European Community Respiratory Health Survey (ECRHS) - Italy. Determinants of bronchial responsiveness in the European Community Respiratory Health Survey in Italy: evidence of an independent role of atopy, total serum IgE levels, and asthma symptoms. *Allergy* 1998; 53: 673–681.
147. Chinn S, on behalf of the European Community Respiratory Health Survey. Individual allergens as risk factors for bronchial responsiveness in the European Community Respiratory Health Survey (ECRHS). *Eur Respir J* 1998; 12: Suppl. 28, 358s.
148. Sunyer J, Antó J, Sabrià J, *et al.* Relationship between serum IgE and airway responsivenss in adults with asthma. *J Allergy Clin Immunol* 1995; 95: 699–705.
149. Burrows B, Sears M, Flannery E, Herbison G, Holdaway M, Silva P. Relation of the course of bronchial responsiveness from age 9 to age 15 to allergy. *Am J Respir Crit Care Med* 1995; 152: 1302–1308.
150. Pattemore P, Holgate S. Bronchial hyperresponsiveness and its relationship to asthma in childhood. *Clin Exp Allergy* 1993; 23: 886–890.
151. Peat J, Tovey E, Mellis C, Leeder S, Woolcock A. Importance of house dsut mite and Alternaria allergens in childhood ashma: an epidemiological study in two climatic regions of Australia. *Clin Exp Allergy* 1993; 23: 812–820.
152. Hargreave F, Ryan G, Thomson N, *et al.* Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance. *J Allergy Clin Immunol* 1981; 68: 347–355.
153. Ferrante E, Corbo G, Valente S, Ciappi G. Associations between atopy, asthma history, respiratory function and non-specific bronchial responsiveness in unselected young asthmatics. *Respiration* 1992; 59: 169–172.
154. Takeda K, Shibasaki M, Takita H. Relation between bronchial responsiveness to methacholine and levels of IgE antibody against *Dermatophagoides farinae* and serum IgE in asthmatic children. *Clin Exp Allergy* 1993; 23: 450–454.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
International Journal for Parasitology 34 (2004) 935–942
INTERNATIONAL Journal for PARASITOLOGY
www.parasitology-online.com

Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria

Sándor Bereczky[a,*], Scott M. Montgomery[b], Marita Troye-Blomberg[c], Ingegerd Rooth[d], Marie-Anne Shaw[e], Anna Färnert[a]

[a]*Infectious Diseases Unit, Karolinska University Hospital, Karolinska Institutet, S-171 76 Stockholm, Sweden*
[b]*Clinical Epidemiology Unit, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden*
[c]*Department of Immunology, Stockholm University, Stockholm, Sweden*
[d]*Nyamisati Malaria Research, Rufiji, National Institute for Medical Research, Dar-es-salaam, Tanzania*
[e]*School of Biology, University of Leeds, Leeds LS2 9JT, UK*

Received 28 January 2004; received in revised form 7 April 2004; accepted 18 April 2004

Abstract

Immunological characteristics were assessed for prospective risk of clinical malaria in a longitudinally followed population in a holoendemic area of Tanzania. Baseline characteristics including crude *Plasmodium falciparum* extract-specific IgE and IgG; total IgE; and parasitological indices, e.g. number of *P. falciparum* clones, were investigated among 700 asymptomatic individuals. Cox regression analysis estimated the risk of succumbing to a new clinical episode during a 40 weeks follow up. High anti-*P. falciparum* IgE levels were associated with reduced risk of acute malaria in all age groups independently of total IgE levels. Statistically significant reduced odds ratio of 0.26 (95% CI, 0.09–0.72, $P = 0.010$) and 0.44 (95% CI, 0.19–0.99, $P = 0.047$) for the two highest fifths, respectively was observed after adjustment for age, sex, total IgE, numbers of parasite clones per infection and HIV-1 seropositivity. In contrast, high levels of malaria specific IgG or total IgE were not associated with reduced risk to succumb to a new clinical episode. A protective effect of asymptomatic multiclonal *P. falciparum* infections was also confirmed. For the first time, anti-malarial IgE levels in asymptomatic individuals in endemic area are found to be associated with reduced risk for subsequent malaria disease. Specific IgE antibodies may play role in maintaining anti-malarial immunity, or indicate other aspects of immune function relevant for protection against malaria.

Keywords: Malaria; *Plasmodium falciparum*; IgE; IgG; Asymptomatic; Protection

1. Introduction

Malaria remains a major threat to global health and yet the mechanisms for protective immunity are still incompletely understood. In areas of high malaria transmission, young children are the most vulnerable to disease and death due to their lack of protective immunity. Prolonged exposure to new inoculations and repeated infections, results in successive acquisition of protective immunity and part of this effect may be associated with controlled persistence of parasites without clinical symptoms.

Mechanisms for malaria immunity are highly complex with involvement of several components of immune function. Antibody-dependent mechanisms are presumed to play an important role in protection, with a wide range of antigen-specific antibodies as well as polyclonal-antibody production. Studies on passively transferred immunoglobulins from immune adults have suggested that IgG antibodies are important in reducing parasite density during clinical malaria disease (Cohen et al., 1961; Sabchareon et al., 1991). There is also accumulating evidence of a protective role for certain IgG subclasses (Aribot et al., 1996; Taylor et al., 1998; Aucan et al., 2000; Ndungu et al., 2002). Although both total and malaria-specific IgE levels are elevated in individuals living in areas of high endemicity (Desowitz, 1989; Perlmann et al., 1994), the role of IgE in malaria is not as clearly established as for other parasites (Hussain and Ottesen, 1986; Hagan et al., 1991; Rihet et al., 1991). The elevated IgE levels seen in severe acute malaria

* Corresponding author. Tel.: +46-8-517-75-281; fax: +46-8-517-76-740.
E-mail address: sandor.bereczky@medks.ki.se (S. Bereczky).

0020-7519/$30.00
doi:10.1016/j.ijpara.2004.04.007

patients indicate a pathogenic role of these antibodies (Perlmann et al., 1994, 1997, 1999, 2000). A protective role for *P. falciparum*-specific IgE has also been proposed (Perlmann et al., 1997, 1999) but this has not been previously demonstrated.

Here, we investigated if plasma levels of the antibody isotypes IgG and IgE are predictive of risk for subsequent acute malaria episodes. In a longitudinal study of a population in Nyamisati village, a highly endemic area the Rufiji Region, coastal Tanzania (Rooth and Bjorkman, 1992), a prospective malariometric survey measured several baseline characteristics, and subsequently recorded episodes of acute malaria over 40 weeks of follow-up. To avoid potential confounding through acute disease activity or treatment, only the individuals with no clinical malaria episodes during the baseline survey, 4 weeks before or 1 week after the survey were included in the analysis. Baseline characteristics included plasma levels of anti-*P. falciparum* (crude) IgE and IgG, total IgE as well as genetic diversity of *P. falciparum* defined as number of clones per infection.

2. Materials and methods

2.1. Study area and population

The study was conducted in Nyamisati village, situated by the Rufiji River Delta, coastal Tanzania. The area is holoendemic for malaria, with perennial transmission and some seasonal fluctuation. A research team, which also provides health care including antimalarial drugs, has lived in the village since 1985, and studied the malaria epidemiology with longitudinal follow up of the population of about 1000 individuals (Rooth and Bjorkman, 1992). In March–April 1999, preceding the heavy rain period, 890 villagers, 1–84 years of age, participated in a malariometric survey including analysis of parasite densities and clinical status.

2.2. Clinical data

Clinical episodes of malaria were detected by a passive case detection system, which is well established in this population with presentation of all fever episodes to the research unit. After diagnosis of malaria by microscopy, free treatment is administered and the episode registered. A clinical malaria episode was defined as >400 parasites/μl, together with reported or confirmed fever within the last day and without signs of other cause of fever (Rooth and Bjorkman, 1992). More than one episode within a period of 2 weeks was considered as a single episode.

At the time of survey 700 individuals were asymptomatic, i.e. there was no record of a clinical malaria episode 4 weeks before and 1 week after the survey. The time period was chosen due to possible confounding effect of sulfadoxine/pyrimethamine (Fansidar®) treatment within 4 weeks prior to survey or potential ongoing disease activity of a clinical episode within the following week.

2.3. Sample collection

Venous blood was collected in EDTA sterile tubes; after centrifugation and separation, plasma and packed cells were stored frozen. All samples were obtained after informed consent of the participants and/or their guardians. The study was approved by the National Institute for Medical Research in Tanzania and by the Ethical Committee at Karolinska Institute (Dnr 00-084).

2.4. Detection of P. falciparum infections

Giemsa stained thick films were analysed by light microscopy at 1000× magnification. Number of parasites per μl of blood was enumerated in 200 microscopic fields corresponding to 0.2 μl blood.

Plasmodium falciparum infections were also detected by nested PCR amplification of the highly polymorphic block 3 region of merozoite surface protein 2 (*msp2*) marker which provides an estimate of the number of concurrent clones (Snounou et al., 1999). DNA was purified from frozen packed red blood cells by phenol–chloroform extraction. Detection of *P. falciparum* infection by this PCR method, i.e. detection of one *msp2* allele or more, yields a higher sensitivity than microscopy with detection of 1–10 parasites per μl whole blood.

2.5. Antibody analysis

Lysates of infected erythrocytes, (*P. falciparum* laboratory line F32), were prepared for determination of anti-malarial IgE and IgG (Perlmann et al., 1994). Briefly, microtiter plates were coated with 50 μl of crude parasite antigen solution (20 μg/ml) overnight at 4 °C, and then blocked with 100 μl of 0.5% BSA in coating buffer for 3 h at 37 °C. For determination of malaria-specific IgE the test plasma were diluted 1:100 and incubated at room temperature overnight for optimal binding. The test plasma, diluted 1:1000 for total IgE and anti-malarial IgG were added to the wells and incubated at 37 °C for 1 h. The secondary antibodies were biotinylated goat anti-human IgE (Vector Laboratories, USA) (1:8000) or goat anti-human IgG (Mabtech, Nacka, Sweden) conjugated to alkaline phosphatase (1:2000), with 1 h incubation at 37 °C. ALP-conjugated Streptavidin (Mabtech, Nacka, Sweden) diluted 1:2000 were added to the biotinylated antibody and incubated under the same conditions. Sera from African donors with high antibody levels and sera from Swedish donors not exposed to malaria were used as positive and negative controls, respectively. The plates were washed four times between each incubation step. The bound secondary

antibody was quantified with p-nitrophenylphosphate (Sigma Diagnostics, USA). The optical density (OD) at 405 nm was determined in a Vmax microplate reader (Molecular Devices, Menlo Park, USA).

2.6. Statistical analysis

Our data was analysed using SPSS software (version 11.5). The analyses excluded individuals with a clinical episode of malaria 4 weeks prior and 1 week after the baseline survey. As we made no assumptions about linearity, the antibody levels were grouped into quintiles of their distributions and the middle fifth was used as the comparison group. Clinical episode of malaria was the dependent variable in Cox-regression analyses. Simultaneous mutual adjustment was made for age, sex, number of *msp2* genotypes, HIV status, antibody levels for specific IgG and IgE, total IgE, as well as parasite density (coded in fifths of the density distribution and a separate, sixth category for no parasites), modelled as fixed covariates. These measures were all coded as series of binary dummy variables. As most symptomatic infections occur in individuals aged 0–16 years, a specific analysis was conducted to confirm the finding among only younger individuals. This included recalculation of the fifths estimating immunoglobulin distributions in this age group.

3. Results

Baseline characteristics were analysed in the 700 individuals (1–84 years, median 23 years) who were asymptomatic at the time of survey. The *P. falciparum* prevalence, including results from microscopy and PCR, was highest in children 0–16 years (59%) and decreased in adults with 32% of individuals over 16 years infected with *P. falciparum*. Including the results from PCR analysis increased *P. falciparum* prevalence by 25% in all age groups. The mean number of clones detected by the PCR genotyping was 2.4, with a range of one to seven clones per isolate (SE = 0.09 clones per infection). More detailed description of the genotyping data will be presented elsewhere.

Prospective risk for malarial disease was based on the first clinical episode after the baseline survey and was assessed using Cox regression analysis. Some 60 individuals experienced at least one clinical episode during the follow up. The clinical malaria episodes were scattered during the 40 weeks period. Table 2 shows the odds ratios from Cox regression for individual baseline characteristics: age, sex, total and anti-*P. falciparum* IgE, number of infecting *msp 2* clones and HIV-1 seropositivity. The odds ratios for risk for clinical malaria episode were highest at younger ages, independent of the other measures. The risk for malarial disease was not statistically significantly associated with sex.

Antibody levels in plasma increased with age but with differing patterns. The overall median value was 1.6 ng/ml for malaria specific IgE, 15.6 μg/ml for specific IgG and 1700 ng/ml for total IgE. Antibody level (median) in negative controls was: 0.8 ng/ml for malaria specific IgE, 1.5 μg/ml for specific IgG and 41 ng/ml for total IgE. Total IgE and anti-*P. falciparum* IgG and IgE levels were grouped into fifths of their distributions (Table 1) and comparisons were made with the middle fifth. Malaria-specific IgE above the middle fifth was associated with a reduced risk for a clinical episode of malaria (Table 2). Statistical significance of these estimates increased after adjustment for the total IgE with odds ratios of 0.26 (95% CI, 0.09–0.72, $P = 0.010$) and 0.44 (95% CI, 0.19–0.99, $P = 0.047$) for the fourth and fifth levels, respectively (Table 2—adjusted values).

When measures of anti-*P. falciparum* IgG and anti-*P. falciparum* IgE were included in the same model with adjustment for all of the potential confounding factors, IgE, but not IgG, was significantly associated with risk of subsequent malarial disease. As anti-malarial IgG was not independently associated with risk of malarial disease it was excluded from the final model to avoid confounding due to co-linearity between IgE and IgG isotypes.

Table 1
Antibody levels grouped into fifths of their distribution

Fifth[a]	Anti-*P. falciparum* IgE		Total IgE		Anti-*P. falciparum* IgG	
	ng/ml[b]	Age[c]	ng/ml[b]	Age[c]	μg/ml[b]	Age[c]
1	0.50 (<0.71)	19	196 (<454)	25	3.1 (<6.0)	12
2	0.93 (0.71–1.25)	23	806 (454–1232)	23	9.0 (6.0–11.9)	17
3	1.54 (1.26–1.93)	26	1701 (1233–2316)	22.5	15.7 (12.0–21.2)	23.5
4	2.47 (1.94–3.42)	22	3150 (2317–4423)	19	28.0 (21.3–40.5)	27
5	5.22 (3.43–20.57)	21	6676 (4424–19275)	21	62.9 (40.6–212.5)	34

Antibody levels (median) in negative controls (Swedish not exposed to malaria) were: anti-*P. falciparum* IgE, 0.8 ng/ml; total IgE, 41 ng/ml; anti-*P. falciparum* IgG, 1.5 μg/ml.

[a] Fifths (140 subjects in each), where 1 is the lowest and 5 is the highest.

[b] Antibody levels, median and range.

[c] Median age (years), within each fifth of the antibody distributions.

Table 2
Baseline characteristics in 700 asymptomatic individuals and prospective risk for clinical malaria episode

Age (years)	Episode after survey[a]		Unadjusted[b]		Adjusted[c]	
	No, *n* (%)	Yes, *n* (%)	Odds ratio	95% CI	Odds ratio	95% CI
≤1	1 (100)	0 (0)	*	*	*	*
2–4	15 (62)	9 (38)	9.95	4.53–21.87	9.37	4.08–21.55
5–7	44 (79)	12 (21)	4.85	2.37–9.92	5.30	2.52–11.14
8–10	55 (87)	8 (13)	2.75	1.21–6.25	3.66	1.52–8.85
11–13	57 (84)	11 (16)	3.52	1.68–7.38	4.43	2.01–9.75
14–16	52 (100)	0 (0)	*	*	*	*
≥17	416 (95)	20 (5)	1.00		1.00	
Sex						
Male	295 (90)	31 (10)	1.00		1.00	
Female	345 (92)	29 (8)	0.83	0.50–1.37	0.96	0.57–1.64
Anti-*Pf.* IgE[d]						
1	123 (88)	17 (12)	1.01	0.52–1.98	1.05	0.51–2.20
2	129 (93)	10 (7)	0.59	0.27–1.28	0.65	0.29–1.47
3	124 (88)	17 (12)	1.00		1.00	
4	135 (96)	5 (4)	0.29	0.11–0.77	0.26	0.09–0.72
5	129 (92)	11 (8)	0.65	0.31–1.39	0.44	0.19–0.99
Total IgE[d]						
1	127 (91)	13 (9)	0.92	0.43–1.96	1.03	0.46–2.31
2	133 (95)	7 (5)	0.49	0.20–1.21	0.53	0.21–1.34
3	126 (90)	14 (10)	1.00		1.00	
4	128 (91)	12 (9)	0.85	0.39–1.83	1.27	0.56–2.91
5	126 (90)	14 (10)	1.01	0.48–2.11	1.91	0.83–4.38
Anti-*Pf.* IgG[d]						
1	114 (81)	26 (19)	2.97	1.39–6.33		
2	131 (94)	9 (6)	0.98	0.39–2.47		
3	131 (94)	9 (6)	1.00			
4	129 (92)	11 (8)	1.23	0.51–2.96		
5	135 (96)	5 (4)	0.55	0.18–1.63		
Nr. clones[e]						
0	396 (91)	39 (9)	0.77	0.39–1.55	0.82	0.40–1.68
1	78 (89)	10 (11)	1.00		1.00	
2–3	107 (96)	5 (4)	0.37	0.13–1.08	0.31	0.10–0.94
≥4	59 (91)	6 (9)	0.79	0.29–2.17	0.46	0.16–1.34
HIV-1						
Seronegative	626 (92)	58 (8)	1.00		1.00	
Seropositive	14 (87)	2 (13)	1.52	0.37–6.21	2.28	0.52–9.96
Total	640	60				

*Not estimated because of empty cells.
[a] Number of individuals with clinical malaria episodes registered within 40 weeks after the survey.
[b] Unadjusted odds ratios from Cox regression analysis.
[c] Odds ratios adjusted for age, sex, anti-*P. falciparum* and total IgE, nr. clones and HIV-1 seropositivity.
[d] Anti-*Plasmodium falciparum*(anti-*Pf.*) and total antibody levels grouped into fifth on their distribution, where 1 is the lowest and 5 is the highest.
[e] Number of parasite clones identified as *msp2* alleles.

In a separate analysis limited to children (0–16years), the two highest fifths of anti-*P. falciparum* IgE levels produced similar adjusted odds ratios of 0.26 (95% CI, 0.08–0.83, $P = 0.024$) and 0.34 (95% CI, 0.12–0.98, $P = 0.046$), respectively. The association between malaria-specific IgE levels and risk for disease was non-linear with a greater protective association for the top two fifths of the distribution. If modelled as an ordinal term in the adjusted model, the trend across the fifths produces an odds ratio of 0.79 (0.64–0.98, $P = 0.030$), despite the non-linearity of the association. Total IgE was not statistically significantly associated with risk for malarial disease when the levels were compared (Table 2) or when trend across levels was estimated, with an odds ratio of 1.20 (0.96–1.52, $P = 0.105$).

The lowest fifth of malaria-specific IgG was associated with an increased risk for a clinical episode of malaria (Table 2-unadjusted values), with odds ratio of 2.97 (95% CI, 1.39–6.33, $P = 0.005$). However, this result no longer remained significant following adjustment for age, with an

odds ratio of 1.91 (95% CI, 0.87–4.20, $P = 0.108$) for the first compared with the middle fifth indicating confounding by age rather than an independent effect. Mutual simultaneous adjustment for the potential confounding factors did not result in any statistically significant associations with clinical malaria. Using Cox regression we found no evidence for statistically significant trend across the groups, either in unjusted or adjusted models. The unadjusted model produced an odds ratio for trend across the groups of 0.88 (0.71–1.10, $P = 0.253$).

When number of *P. falciparum* clones was assessed, the risk of subsequent episode was found to be lower in individuals with multiclonal infections. The odds ratios were of 0.31 (95% CI, 0.10–0.94, $P = 0.038$) for two to three clones and 0.46 (95% CI, 0.16–1.34, $P = 0.154$) for four or more clones, compared with having one single clone. The risk was, however, also reduced in subject with no clones, i.e. absence of detectable parasites, with an odds ratio of 0.82 (95% CI, 0.40–1.68, $P = 0.588$) compared with having one clone (Table 2). In the children (age under 17 years) the odds ratios are 0.26 (95% CI, 0.08–0.90, $P = 0.033$) for two to three clones, 0.32 (95% CI, 0.10–1.06, $P = 0.062$) for four or more clones and 0.66 (95% CI, 0.28–1.58, $P = 0.354$) in subjects with no clones.

The risk for malarial disease was not statistically significantly associated with HIV-1 seropositivity (Table 2). However, a separate analysis limited to children (0–16 years) revealed an increased risk for HIV-1 seropositives, producing odds ratio of 11.65 (95% CI, 1.12–121.01, $P = 0.040$).

Parasite density among these asymptomatic individuals, in neither the univariate nor the multivariate analysis, was statistically significantly associated with risk of subsequent clinical malaria and adjustment for parasite density did not statistically significantly alter any of the other associations with risk for malarial disease.

4. Discussion

In this study we investigated factors among subjects free of acute malaria, associated with the risk of subsequent acute malaria episodes. After adjustment for multiple potential confounding factors, we observed independent associations of older age and higher levels of anti-*P. falciparum* IgE with a reduced risk of clinical malarial disease. The non-linear association of malaria-specific IgE with disease risk may reflect antagonistic influences.

The importance of anti-malarial IgE in protective malaria immunity has long been proposed (Perlmann et al., 1997, 1999) but not previously demonstrated. IgE antibody levels have mainly been investigated during the acute phase of disease, comparing patterns in mild and severe, e.g. cerebral malaria (Perlmann et al., 1994, 1997, 2000), or correlating *P. falciparum*-IgE levels and placental parasitemias (Desowitz et al., 1993; Maeno et al., 1993). To our knowledge, the present study is the first to prospectively assess IgE antibody levels in asymptomatic subjects and describe its association with the risk for subsequent malarial disease.

Several methodological considerations, in addition to the large sample size, represent advantages in this assessment of predictive markers for anti-malarial immunity. Firstly, baseline measurements of antibody levels and other factors were collected in asymptomatic individuals: thus, these measures were unaffected by ongoing or recent malarial disease or treatment, which may result in temporally affected serum levels. While this was necessary to avoid confounding by disease activity, some of the most susceptible individuals may be excluded from the analysis, such that our results could underestimate some associations. Secondly, the baseline measurements were collected prospectively and risk of subsequent disease was studied with recording of each clinical episode of malarial disease in the entire study population, so retrospective recording did not introduce error. Thirdly, we used Cox regression analysis, providing a sensitive measure of risk taking duration of follow-up into account. Multiple simultaneous adjustment were made for factors, such as age, sex, HIV status and parasite densities, thus eliminating many of the problems associated with residual confounding that may influence results.

Our data revealed that high levels of malaria specific IgE were associated with reduced risk for subsequent clinical episodes of malaria. The effect was most prominent in the two highest fifths of the distribution, when compared with the middle levels. Even though a statistically significant trend across the categories was established, this relationship was non-linear, which may reflect that the two lowest levels lie around the threshold of detection of the assay. The non-linearity even seen within the highest anti-*P. falciparum* levels may reflect opposing elements of protection against malaria (discussed below). Among children, who are most vulnerable to infection, higher levels of malaria-specific IgE were associated with a statistically significantly reduced risk of acute malaria. This indicates that the association of IgE with protection from malaria may occur in all age groups, but this is also evidence of its potential importance during the years of greatest vulnerability to acute malarial disease. Total IgE was not associated with reduced risk for disease, and the association found for anti-*P. falciparum* IgE was not solely a function of high total IgE levels. When modelled together with the malaria-specific IgG and the other potential confounding factors, specific IgE was statistically significantly associated with risk of malarial disease, while specific IgG was not.

In univariate analysis, the risk for subsequent malarial disease was highest in subjects with the very low levels of malaria-specific IgG. However, this association is due to confounding by age as demonstrated by the elimination of this association by adjustment for age alone. This is because there is an almost linear increase of malaria-specific IgG

levels with age and the risk of malarial disease decreases with age. Levels of IgG specific to crude *P. falciparum* lysates were not found to be predictive of subsequent malaria episodes, even where trend across the categories was estimated to increase statistical power. The reasons for not finding an association between anti-malarial IgG and protection may be because these antibodies contain many more antigenic specificities which can not be distinguished in our ELISA using a crude *P. falciparum* antigen extract. Another factor could be the distribution of different IgG subclasses. Several studies have reported associations between different IgG subclass responses to specific antigens and clinical protection, e.g. IgG1 (Ndungu et al., 2002), IgG2 (Aucan et al., 2000) and IgG3 (Taylor et al., 1998; Aucan et al., 2000; Oeuvray et al., 2000). The overall malaria specific IgG levels were however not found to be protective (Aribot et al., 1996; Ndungu et al., 2002), which is consistent with our findings in the present study. Analysis of total IgG levels was not included in the assessment since these measurements may produce poor estimates due to aggregation of antibodies at very high concentrations. A potential effect of different IgG subclasses and antigenic specificities needs further investigation in this population.

Persistence of *P. falciparum* infections even at low levels may be an important feature in acquisition of protective immunity in individuals in malaria endemic areas (Smith et al., 1999). Presence of *P. falciparum* parasites per se or total number, i.e. parasite density was not a confounder for the associations of antibody levels with risk for subsequent malaria disease. Fluctuations in number of parasites in the peripheral blood at different time points may however restrict the value of single measurements of parasite densities in asymptomatic individuals (Farnert et al., 1997; Delley et al., 2000). In contrast, the genetic diversity of *P. falciparum*, i.e. multi-clonal infections, was associated with reduced risk for subsequent malaria disease, consistent with previous reports (Al-Yaman et al., 1997; Farnert et al., 1999; Smith et al., 1999), suggesting the importance of polyclonal antigen stimulation. The association between number of *P. falciparum* clones and risk of subsequent acute malaria was however not straightforward, with both no detectable clones and multiple clones associated with protection, when compared with a single clone infection. This may be because anti-malarial immunity is based on an equilibrium, where controlled maintenance of parasites is associated with protection. This may help to explain the non-linear association with other factors such as specific IgE level: if IgE is protective against infection this is a benefit, but too high a level may prevent an adequate level of controlled parasite persistence to fully maintain functional immunity. The different elements of anti-malarial immunity do not operate in unison and this highlights the complexity of malaria immunity.

Longitudinal data of malaria-specific IgE levels over time within asymptomatic individuals has to our knowledge not been reported. In other parasitic infections, e.g. *Dracunculus medinensis* (Bloch and Simonsen, 1998) and *Wuchereria bancrofti* (Jaoko et al., 2001), levels have been shown to be stable within the individual, with no seasonal or patency-dependent variation in the levels of parasite-specific IgE. The IgE antibodies detected in our assay correspond to the free fraction of IgE with short half-life (about 2.5 days) which may fluctuate over time, thus the one-time measurement may rather underestimate an association between malaria-specific IgE and protection against acute malarial disease.

The potentially protective mechanisms of anti-malarial IgE need to be clarified. They might act in concert with cell types equipped with receptors for IgE and thereby mediate effector mechanisms as shown for helminthic infections (Pirron et al., 1990; van der Heijden, 1993). However, cell bound IgE can also be involved in presenting antigen to T cells (Heyman, 2002). In addition IgE, in the form of immune complexes, is a potent inducer of tumor-necrosis factor (TNF) in human monocytes (Dugas et al., 1995; Perlmann et al., 1997), a factor involved in both immune protection and pathogenesis of malaria (Kwiatkowski et al., 1990; Perlmann et al., 1996). The lack of association between total IgE and reduced risk for subsequent infection, favours an antigen-specific protective mechanism. How can anti-malarial IgE be involved in both protection and pathogenesis of malaria? The result of IgE responses is probably a combination of induction and host genetic factors (Rosenwasser et al., 1995; Hill, 1999; Perlmann et al., 1999; Troye-Blomberg, 2002). If one of the role of IgE is induction of TNF, over-producers of TNF may have an increased risk of succumbing to severe disease while in individuals with low TNF production IgE antibodies might help to protect against disease (Kwiatkowski et al., 1990; Knight et al., 1999; McGuire et al., 1999). Moreover, the role of IgE is probably different in the maintenance of protective immunity in asymptomatic individuals, compared to the immune activation in acute clinical malaria. Further studies are thus needed to clarify the role of anti-malarial IgE in immune reactions against malaria.

Asymptomatic individuals with higher levels of anti-malarial IgE were less likely to exhibit a clinical episode of malaria, independent of age and other potential confounding factors. Neither high levels of total IgE, nor malaria-specific IgG were found to be predictive of protection. Malaria-specific IgE antibody levels may represent a useful and readily available indicator of susceptibility to subsequent malarial disease in asymptomatic individuals.

Acknowledgements

We are most grateful to the villagers and research team in Nyamisati; Hedvig Perlmann for methodological expertise and comments, Danielle Carpenter, Hind Abushama and Margareta Hagstedt for excellent technical assistance; Professor Anders Björkman and Dr Ze Pedro Gil for

remarks on the manuscript. This work was supported by the Swedish International Development Cooperation Agency—SAREC (Project grant SWE 2002-066 and 1995-117), the Swedish Medical Research Council and the Bergvall's Foundation.

References

Al-Yaman, F., Genton, B., Reeder, J.C., Anders, R.F., Smith, T., Alpers, M.P., 1997. Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. Trans. R. Soc. Trop. Med. Hyg. 91, 602–605.

Aribot, G., Rogier, C., Sarthou, J.L., Trape, J.F., Balde, A.T., Druilhe, P., Roussilhon, C., 1996. Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). Am. J. Trop. Med. Hyg. 54, 449–457.

Aucan, C., Traore, Y., Tall, F., Nacro, B., Traore-Leroux, T., Fumoux, F., Rihet, P., 2000. High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to *Plasmodium falciparum* malaria. Infect. Immun. 68, 1252–1258.

Bloch, P., Simonsen, P.E., 1998. Immunoepidemiology of *Dracunculus medinensis* infections II. Variation in antibody responses in relation to transmission season and patency. Am. J. Trop. Med. Hyg. 59, 985–990.

Cohen, S., McGregor, I.A., Carrington, S., 1961. Gamma-globulin and acquired immunity to human malaria. Nature 192, 733–737.

Delley, V., Bouvier, P., Breslow, N., Doumbo, O., Sagara, I., Diakite, M., Mauris, A., Dolo, A., Rougemont, A., 2000. What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. Trop. Med. Int. Health 5, 404–412.

Desowitz, R.S., 1989. *Plasmodium*-specific immunoglobulin E in sera from an area of holoendemic malaria. Trans. R. Soc. Trop. Med. Hyg. 83, 478–479.

Desowitz, R.S., Elm, J., Alpers, M.P., 1993. *Plasmodium falciparum*-specific immunoglobulin G (IgG), IgM, and IgE antibodies in paired maternal-cord sera from east Sepik Province, Papua New Guinea. Infect. Immun. 61, 988–993.

Dugas, B., Mossalayi, M.D., Damais, C., Kolb, J.P., 1995. Nitric oxide production by human monocytes: evidence for a role of CD23. Immunol. Today 16, 574–580.

Farnert, A., Snounou, G., Rooth, I., Bjorkman, A., 1997. Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. Am. J. Trop. Med. Hyg. 56, 538–547.

Farnert, A., Rooth, I., Svensson, A., Snounou, G., Bjorkman, A., 1999. Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. J. Infect. Dis. 179, 989–995.

Hagan, P., Blumenthal, U.J., Dunn, D., Simpson, A.J., Wilkins, H.A., 1991. Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. Nature 349, 243–245.

Heyman, B., 2002. IgE-mediated enhancement of antibody responses: the beneficial function of IgE? Allergy 57, 577–585.

Hill, A.V., 1999. The immunogenetics of resistance to malaria. Proc. Assoc. Am. Physicians 111, 272–277.

Hussain, R., Ottesen, E.A., 1986. IgE responses in human filariasis. IV. Parallel antigen recognition by IgE and IgG4 subclass antibodies. J. Immunol. 136, 1859–1863.

Jaoko, W.G., Simonsen, P.E., Meyrowitsch, D.W., Pedersen, E.M., Rwegoshora, R.T., Michael, E., 2001. *Wuchereria bancrofti* in a community with seasonal transmission: stability of microfilaraemia, antigenaemia and filarial-specific antibody concentrations. Ann. Trop. Med. Parasitol. 95, 253–261.

Knight, J.C., Udalova, I., Hill, A.V., Greenwood, B.M., Peshu, N., Marsh, K., Kwiatkowski, D., 1999. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat. Genet. 22, 145–150.

Kwiatkowski, D., Hill, A.V., Sambou, I., Twumasi, P., Castracane, J., Manogue, K.R., Cerami, A., Brewster, D.R., Greenwood, B.M., 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet 336, 1201–1204.

Maeno, Y., Steketee, R.W., Nagatake, T., Tegoshi, T., Desowitz, R.S., Wirima, J.J., Aikawa, M., 1993. Immunoglobulin complex deposits in *Plasmodium falciparum*-infected placentas from Malawi and Papua New Guinea. Am. J. Trop. Med. Hyg. 49, 574–580.

McGuire, W., Knight, J.C., Hill, A.V., Allsopp, C.E., Greenwood, B.M., Kwiatkowski, D., 1999. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J. Infect. Dis. 179, 287–290.

Ndungu, F.M., Bull, P.C., Ross, A., Lowe, B.S., Kabiru, E., Marsh, K., 2002. Naturally acquired immunoglobulin (Ig)G subclass antibodies to crude asexual *Plasmodium falciparum* lysates: evidence for association with protection for IgG1 and disease for IgG2. Parasite Immunol. 24, 77–82.

Oeuvray, C., Theisen, M., Rogier, C., Trape, J.F., Jepsen, S., Druilhe, P., 2000. Cytophilic immunoglobulin responses to *Plasmodium falciparum* glutamate-rich protein are correlated with protection against clinical malaria in Dielmo. Senegal. Infect. Immun. 68, 2617–2620.

Perlmann, H., Helmby, H., Hagstedt, M., Carlson, J., Larsson, P.H., Troye-Blomberg, M., Perlmann, P., 1994. IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: association of high IgE levels with cerebral malaria. Clin. Exp. Immunol. 97, 284–292.

Perlmann, P., Perlmann, H., Flyg, B.W., Hagstedt, M., Elghazali Worku, S., Fernandez, V., Rutta, A.S., Troye-Blomberg, M., 1997. Immunoglobulin E, a pathogenic factor in Plasmodium falciparum malaria. Infect. Immun. 65, 116–121.

Perlmann, P., Perlmann, H., Blomberg, M.T., 1996. IgE, TNF in malaria infection. Protection and pathogenicity: two sides of the same coin. Immunologist 4/5, 179–184.

Perlmann, P., Perlmann, H., ElGhazali, G., Blomberg, M.T., 1999. IgE and tumor necrosis factor in malaria infection. Immunol. Lett. 65, 29–33.

Perlmann, P., Perlmann, H., Looareesuwan, S., Krudsood, S., Kano, S., Matsumoto, Y., Brittenham, G., Troye-Blomberg, M., Aikawa, M., 2000. Contrasting functions of IgG and IgE antimalarial antibodies in uncomplicated and severe *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg. 62, 373–377.

Pirron, U., Schlunck, T., Prinz, J.C., Rieber, E.P., 1990. IgE-dependent antigen focusing by human B lymphocytes is mediated by the low-affinity receptor for IgE. Eur. J. Immunol. 20, 1547–1551.

Rihet, P., Demeure, C.E., Bourgois, A., Prata, A., Dessein, A.J., 1991. Evidence for an association between human resistance to *Schistosoma mansoni* and high anti-larval IgE levels. Eur. J. Immunol. 21, 2679–2686.

Rooth, I., Bjorkman, A., 1992. Fever episodes in a holoendemic malaria area of Tanzania: parasitological and clinical findings and diagnostic aspects related to malaria. Trans. R. Soc. Trop. Med. Hyg. 86, 479–482.

Rosenwasser, L.J., Klemm, D.J., Dresback, J.K., Inamura, H., Mascali, J.J., Klinnert, M., Borish, L., 1995. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin. Exp. Allergy 25(Suppl 2), 74–78.discussion 95–96.

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-Tayoun, H., Chantavanich, P., Foucault, C., Chongsuphajaisiddhi, T., Druilhe, P., 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am. J. Trop. Med. Hyg. 45, 297–308.

Smith, T., Felger, I., Tanner, M., Beck, H.P., 1999. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. Trans. R. Soc. Trop. Med. Hyg. 93(Suppl 1), 59–64.

Snounou, G., Zhu, X., Siripoon, N., Jarra, W., Thaithong, S., Brown, K.N., Viriyakosol, S., 1999. Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. Trans. R. Soc. Trop. Med. Hyg. 93, 369–974.

Taylor, R.R., Allen, S.J., Greenwood, B.M., Riley, E.M., 1998. IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. Am. J. Trop. Med. Hyg. 58, 406–413.

Troye-Blomberg, M., 2002. Genetic regulation of malaria infection in humans. Chem. Immunol. 80, 243–252.

van der Heijden, F.L., Joost van Neerven, R.J., van Katwijk, M., Bos, J.D., Kapsenberg, M.L., 1993. J. Immunol. 150, 3643–3650.

Clinical and Experimental Allergy, 1999, Volume 29, pages 28–34

Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community

CH. BRAUN-FAHRLÄNDER*, M. GASSNER†, L. GRIZE*, U. NEU‡, F. H. SENNHAUSER§, H. S. VARONIER¶, J. C. VUILLE**, B. WÜTHRICH†† and THE SCARPOL TEAM

**Institute of Social and Preventive Medicine, University of Basel, †School Health Service, Grabs, ‡Institute of Geography, University of Bern, §Paediatric Clinic of the University Hospital of Zürich, ¶Allergy Clinic, Paediatric University Hospital, Geneva, **Department of Public Health, City of Bern, ††Department of Dermatology, University Hospital, Zürich, Switzerland*

Summary

Introduction Lower prevalence rates of allergic diseases in rural as compared with urban populations have been interpreted as indicating an effect of air pollution. However, little is known about other factors of the rural environment which may determine the development of atopic sensitization and related diseases.
Objective The authors tested the hypothesis that children growing up on a farm were less likely to be sensitized to common aerollergens and to suffer from allergic diseases than children living in the same villages but in nonfarming families.
Materials and methods Three age groups of schoolchildren (6–7 years, 9–11 years, 13–15 years) living in three rural communities were included in the analyses. An exhaustive questionnaire was filled in by 1620 (86.0%) parents. A blood sample was provided by 404 (69.3%) of the 13–15 year olds to determine specific IgE antibodies against six common aeroallergens.
Results Farming as parental occupation was reported for 307 children (19.0%). After adjustment for potential covariates such as family history of asthma and allergies, parental education, number of siblings, maternal smoking, pet ownership, indoor humidity and heating fuels, farming as parental occupation was significantly associated with lower rates of sneezing attacks during pollen season (adjusted OR 0.34, 95% CI 0.12–0.89) and atopic sensitization (adjusted OR 0.31, 95% CI 0.13–0.73) whereas the association with wheeze (adjusted OR 0.77 95% CI 0.38–1.58) and itchy skin rash (adjusted OR 0.86, 95% CI 0.49–1.50) was not statistically significant. The risk of atopic sensitization was lower in children from full-time farmers (adjusted OR 0.24, 95% CI 0.09–0.66) than from part-time farmers (adjusted OR 0.54, 95% CI 0.15–1.96).
Conclusion Factors directly or indirectly related to farming as parental occupation decrease the risk of children becoming atopic and developing symptoms of allergic rhintis.

Keywords: hay fever, allergic sensitization, farming, children

Clinical and Experimental Allergy, Vol. 29, pp. 28–34. Submitted 29 June 1998; revised 4 August 1998; accepted 14 August 1998.

Introduction

Several recent population studies demonstrated lower prevalence rates of allergic diseases in rural as compared with urban populations [1–3], which has been interpreted as indicating an effect of air pollution. However, the role of air pollution in the development of allergic diseases and sensitization to inhalant allergens is still controversial. The principal literature arguing for a significant role of air

Correspondence: Dr med. Ch. Braun-Fahrländer, Institut für Sozial-und Präventivmedizin der Universität Basel, Steinengraben 49, CH-4051 Basel, Switzerland.

pollution comprises toxicological studies showing increased primary IgE responses in animals exposed acutely to relatively high levels of pollutants, concomitantly with allergen aerosols [4,5]. Several comparative epidemiological studies in Eastern and Western Europe [6,7], and the authors' own recently conducted Swiss Study on Childhood Allergy and Respiratory symptoms with respect to air Pollution (SCARPOL) could not demonstrate an association between long-term air pollution and the prevalence of allergic diseases [8,9].

The clinical observation of one of the SCARPOL investigators that allergic diseases such as hay fever were extremely rare among farmers' children [10] and a report from rural South Bavaria relating lower rates of allergic diseases to the use of coal and wood stoves for heating [11] led us to investigate whether factors of the rural environment other than air pollution were associated with lower rates of allergic disease and sensitization rates, and thus might explain the lower prevalence rates of allergies in rural communities.

The present study was part of the ongoing Swiss multicentre study SCARPOL. The analyses focused on schoolchildren living in three rural communities. Little is known about factors of the rural environment determining the development of atopic sensitization and related diseases. Therefore, the hypothesis that children growing up on a farm were less likely to be sensitized to common aerollergens and to suffer from allergic diseases than children living in the same villages but in nonfarming families was tested.

Methods

Study organization and participation rate

The Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution (SCARPOL) is a multicentre study designed to investigate associations between air pollution and respiratory and allergic symptoms in school children living in 10 communities [8]. It was first conducted during the school year 1992/1993 and repeated with identical methods as part of a environmental health monitoring program in 1995/96.

The study was organized within the framework of the School Health Services thereby taking advantage of an existing structure of the Health System. The children visit the School Health Services routinely at the age of 6–7 years (1st grade), 9–11 years (4th grade) and 13–15 years (8th grade).

The present analyses are restricted to the children living in the three rural SCARPOL communities (Grabs, Langnau, Payerne and surrounding villages). To avoid problems relating to language proficiency and literacy, the sample is restricted to Swiss nationals. Since there were no systematic differences in prevalence rates of respiratory and allergic symptoms or in atopic sensitization rates between the two surveys, data of both surveys were pooled. The second survey included only children who did not take part in the first survey.

All children visiting the School Health Services in their respective communities were invited to participate in the study. A total of 1620 (86.0%) parents filled in the questionnaire and allowed their children to participate in the study.

In addition, the 13–15 year olds were asked to provide a blood sample for specific IgE-testing, and 404 (69.3%) of the invited adolescents accepted. The prevalence rates of respiratory and allergic symptoms did not differ between those who provided blood samples and those who refused.

The study protocol was approved by the Ethics Committee of the University of Bern.

Questionnaire

The detailed questionnaire completed by the parents included questions on respiratory and allergic symptoms, family history of respiratory and allergic diseases, number of siblings, parental education, indoor fuels, passive smoking, indoor humidity, pet ownership, and study area. The core questions on asthma and allergy of the International Study on Childhood Asthma and Allergy (ISAAC) [12] were incorporated into the questionnaire.

Parents had to indicate whether the family was running a farm and if so, whether this was a full-time or part-time activity.

Eight respiratory and allergic illness and symptom responses obtained from the questionnaire were considered: repeated episodes of cough, and bronchitis occurring during the past year, current wheeze, asthma, sneezing attacks during pollen season, hay fever, itchy skin rash, and eczema.

Wheeze was defined as a positive response to the question: 'Has your child had wheezing or whistling in the chest in the last 12 months?' *S*neezing during pollen season was an affirmative answer to the question: 'In the past 12 months, has your child had a problem with sneezing or a runny or blocked nose when he/she did not have a cold or the flu?' combined with the indication of the symptoms occurring during the months of April through September (pollen season), a symptom that showed high validity in the authors' recent validation study [13]. Itchy skin rash was defined as a positive response to the question: 'Has your child ever had an itchy rash which was coming and going for at least 6 months?' In addition, affirmative answers to the questions: 'Has your child ever had asthma, hay fever or eczema?' were considered.

Serological tests

Blood samples (10 cm^3) were taken and the serum separated by spinning 10 min at 1000 ***g***. The samples were kept frozen and sent to the allergy laboratory of the Department of Dermatology in Zürich.

The multiscreen test, SX_1 (screening test for eight common inhalative allergens, Pharmacia,) was performed with all serum samples by CAP-FEIA technology. If the SX_1 test was positive, the specific IgE levels to six allergens (timothy grass, birch, mugwort, house dust mite, cat and dog dander) were determined by CAP-FEIA. The cutoff for a positive result was defined as a specific IgE concentration ≥ 0.69 KU/L (CAP class ≥ 2). In addition to the SX_1 test, positive reactions to any of the outdoor allergens (timothy grass, birch, mugwort) and to any of the indoor allergens

Table 1. Characteristics of the study population according to farming as parental occupation

	Total study population $N = 1620$ (n,%)	Parental occupation		P value (χ^2 test)
		Farming $N = 307$ (n,%)	Non-Farming $N = 1313$ (n,%)	
Sex				
Boys	844/52.1	161/52.4	683/52.0	n.s.
Girls	776/47.9	146/47.6	630/48.0	
Age group				
6–7	555/34.3	108/35.3	447/34.0	
9–12	481/29.7	83/27.1	398/30.3	n.s.
13–15	583/36.0	115/37.6	468/35.6	
(Missing: 1)				
Parental education				
Low	135/8.3	66/21.5	69/5.3	
Medium	952/58.8	201/65.5	751/57.2	<0.001
High	469/29.0	33/10.7	436/33.2	
(Missing: 64)				
Number of siblings				
None	97/6.0	9/2.9	88/6.7	
1	677/41.8	55/17.9	622/47.4	
2	508/31.4	94/30.6	414/31.5	<0.001
3+	257/15.9	108/35.2	149/11.3	
(Missing: 81)				
Mother smoking	375/23.2	55/18.0	320/24.4	0.015
(Missing: 12)				
Father smoking	473/29.2	88/28.7	385/29.3	n.s.
(Missing: 12)				
Furred pets	1041/64.3	268/87.3	773/58.9	0.001
Pets in bedroom	430/26.5	56/18.2	374/28.5	0.001
Indoor humidity	353/21.8	87/28.3	266/20.3	0.02
Heating				
Central	1284/79.3	206/67.1	1078/82.1	
Single space gas/oil	30/1.9	6/2.0	24/1.8	
Electric	38/2.4	7/2.3	31/2.4	<0.001
Wood/coal	187/11.5	72/23.5	115/8.8	
(Missing: 81)				
Family history of asthma	265/16.4	38/12.4	227/17.3	0.05
(Missing: 27)				
Family history of hay fever	425/26.2	39/12.7	386/29.4	0.001
Family history of eczema	415/25.6	65/21.2	350/26.7	0.05

(house dust mite, cat and dog dander) were considered. Because of the small numbers, it was not possible to further analyse individual allergens.

Ambient monitoring

Air pollutants and meteorological parameters were monitored by local authorities and the Swiss Institute of Meteorology, Zürich, respectively. Annual mean concentrations of air pollutants such as NO_2, PM_{10} or ozone were similar in the three communities, ranging from 13.2 to 19.2 $\mu g/m^3$ for PM_{10}, 19.7–21.9 $\mu g/m^3$ for NO_2, and 43–50 $\mu g/m^3$ for ozone. Mean annual temperature ranged from 8 to 10 °C, and mean relative humidity from 73.3% to 81.1%. The study communities were situated between 451 and 557 m above sea-level.

Statistical analyses

Symptom prevalence and atopic sensitization rates were analysed for the total study population and stratified by farming as parental occupation. Since farming families may differ in many respects from nonfarming families, socioeconomic, personal, and indoor characteristics were analysed according to farming as parental occupation. Differences between categorical variables were assessed by the χ^2 test. Logistic regression analysis was used to evaluate whether the association between farming as a parental occupation and allergic symptoms, and sensitization rates persisted after adjustment for potential confounders. The models included age, sex, parental education, a family history of asthma, a family history of hay fever or eczema, number of siblings, maternal smoking, pet ownership, indoor humidity, and heating fuels. Crude and adjusted odds ratios (OR) and the corresponding 95% confidence intervals (CI) were computed.

Results

Study population

The study population consisted of three age groups of school children: 555 (34.3%) from 1st grade, 481 (29.7%) from 4th grade and 583 (36.0%) from 8th grade. Their respective mean ages were 6.5 years (SD ± 0.5), 10.0 years (SD ± 0.7) and 14.1 years (SD ± 0.7). Eight hundred and forty-four

Table 2. Association of respiratory and allergic symptoms[1] and allergic sensitization with farming as parental occupation

	Total study population (*n*,%)	Symptom prevalence according to parental occupation		Association with farming as parental occupation	
		Farming (*n*,%)	Non-Farming (*n*,%)	Crude OR (95% CI)	Adjusted[3] OR (95% CI)
*Questionnaire (*N = *1620)*					
Repeated cough	594/36.7	103/33.6	491/37.4	0.85 (0.65–1.10)	0.90 (0.63–1.29)
Bronchitis	156/9.6	31/10.1	125/9.5	1.07 (0.71–1.62)	1.37 (0.77–2.40)
Wheeze	135/8.3	16/5.2	119/9.1	0.55 (0.33–0.94)	0.77 (0.38–1.58)
Asthma (ever)	150/9.3	24/7.8	126/9.6	0.80 (0.51–1.26)	1.17 (0.64–2.13)
Sneezing during pollen season	125/7.7	8/2.6	117/8.9	0.27 (0.14–0.54)	0.34 (0.12–0.89)
Hay fever (ever)	197/12.2	22/7.2	175/13.3	0.50 (0.32–0.79)	0.89 (0.49–1.59)
Itchy skin rash (ever)	193/12.0	27/8.9	166/12.7	0.67 (0.41–1.02)	0.86 (0.49–1.50)
Eczema (ever)	305/18.8	48/15.6	257/19.6	0.76 (0.54–1.07)	1.15 (0.74–1.81)
Serological tests[2] *(*N = *404)*					
Positive SX_1 test (CAP-class ≥ 2)	139/34.4	16/18.6	123/38.7	0.33 (0.18–0.59)	0.31 (0.13–0.73)
Specific IgE's to outdoor allergens (CAP class ≥ 2)	119/29.5	15/17.4	104/32.7	0.43 (0.24–0.78)	0.38 (0.16–0.87)
Specific IgE's to indoor allergens (CAP class ≥ 2)	81/20.1	4/4.7	77/24.2	0.15 (0.06–0.38)	0.15 (0.04–0.57)

[1]during the past 12 months if not otherwise specified. [2]318 serological tests were done in children from nonfarming families, 86 in farmers' children. [3]The logistic regression model included the following variables: age, sex, parental education, a family history of asthma, hay fever, eczema, number of siblings, maternal smoking, pet ownership, indoor humidity, study area and heating fuels.

(52.1%) were boys and 776 (47.9%) were girls. Farming as parental occupation was reported for 307 (19.0%) children, 220 (13.6%) as a full-time activity, and 87 (5.4%) as a part-time activity.

The characteristics of the total study population according to farming as a parental occupation are given in Table 1. The socioeconomic and home characteristics of farming families differed in many respects from nonfarming families living in the same rural communities. Farming families were of lower socioeconomic status, had more children, often reported more humidity spots or visible molds in their home, heated their homes more often with traditional heating systems using mainly coal and wood and were more likely to keep furred pets, but these pets were less often allowed in the child's bedroom. Mothers in farming families were less likely to smoke and a family history of asthma, hay fever or eczema was reported less often in these families.

The bivariate analyses of respiratory and allergic symptoms and sensitization rates showed that significantly lower rates of current wheeze (5.2% vs 9.5%), sneezing attacks during the pollen season (2.6% vs 8.6%), and hay fever (7.2% vs 13.3%) were reported for farmers' children as compared with children from a nonagricultural environment, whereas the association with a reported diagnosis of asthma, itchy skin rash and eczema did not reach statistical significance (Table 2). The prevalence of bronchitis and repeated cough during the past year was similar in children from farming and nonfarming families. Children from farming families were significantly less likely to have a positive SX_1 test (18.6% vs 38.7%), and specific IgEs to outdoor allergens (17.4% vs 32.7%) and to indoor allergens (4.7% vs 24.2%) than children from nonfarming families.

Since many of the characteristics in which farming and nonfarming families differed have been associated with an increased risk for allergic disease (e.g. family history of asthma, hay fever or eczema), or a lower risk of allergies, such as the number of siblings and the use of wood for heating, the authors evaluated whether these potential confounders would affect the observed association of atopic sensitization and related diseases and farming as parental occupation. The covariates listed in Table 1 were incorporated into a logistic regression model (Table 2). Adjustment for covariates reduced the association of farming as parental occupation with current wheeze, hay fever and itchy skin rash, but was essentially unchanged and remained statistically significant for reported sneezing attacks during the pollen season (adjusted OR 0.34, 95% CI 0.12–0.89) and for a positive SX_1-test (adjusted OR 0.31, 95% CI 0.13–0.73). The low risk of a sensitization to outdoor and indoor allergens associated with farming as parental occupation was also unaffected by the adjustment of covariates.

The association between reported sneezing attacks and a positive SX_1 test with farming as parental occupation for children from full-time and part-time farmers as compared with nonfarming families was separately assessed. The adjusted ORs for sneezing attacks during the pollen season associated with part-time and full-time farming were 0.31 (95% CI 0.06–1.35) and 0.36 [0.10–1.22], respectively. For a positive SX_1 test the adjusted ORs were 0.54 [0.15–1.96] and 0.24 [0.09–0.66], respectively, indicating a gradient in atopic sensitization from nonfarming to full-time farming as parental occupation.

Discussion

The results of this analysis suggest that factors directly or indirectly related to farming as parental occupation reduce the risk of producing specific IgE antibodies to aeroallergens and of developing the clinical symptoms of allergic rhinitis. The amount of risk reduction for the development of atopic sensitization determined in this study is similar to the one observed in children from East Germany as compared with West Germany [6]. However, the children of the present study are living in the same villages.

Lower rates of sensitization to pollen and animal dander and self-reported hay fever have been observed in occupational studies of adult farmers [14–17]. The results of the present study are also supported by a study in Swedish conscripts [18] and a more recent study among Finnish university students demonstrating lower prevalence rates of self-reported allergic rhinitis in students who had been raised on a farm as compared with students from a nonagricultural environment [18,19].

Children from rural South Bavaria in Germany had lower rates of hay fever, atopic sensitization and bronchial hyperresponsiveness when living in a home where coal or wood was used for heating compared with children from homes with other heating systems [11]. In the present study, coal or wood as heating fuel was also associated with a lower risk of atopic sensitization (adjusted OR 0.77, 95% CI 0.35–1.64), but the effect of farming as a parental occupation was stronger. Although no information on parental occupation was available in the German study, it seems quite likely that most of the families with traditional heating systems were farmers.

A serious concern when interpreting the results of the present study is selection bias. If farmers suffering from allergic diseases such as hay fever move into other jobs a 'healthy or nonallergic' farmer effect would occur resulting in lower rates of atopic disease in the farming parents of this study population. This has indeed been observed in the authors' data. However, the association between atopic sensitization and farming as parental occupation remained significant after adjustment for family history of allergic diseases. In addition, the observation of a gradient in the prevalence of atopic sensitization from full-time to part-

time and nonfarming subjects argues against selection bias. Many farmers had to give up farming as a full-time activity and move to other jobs because of economic constraints during recent years, and not because one of the children developed hay fever. Usually, the family remains in the farm house and does some farming as part-time activity. Thus, full-time and part-time farming may indicate different degrees of exposure to a factor which interferes with the production of specific IgE antibodies to allergens.

Farms are rather small in these communities, usually managed by one family and farming activities include dairy farming and agriculture. Thus, one might speculate that children living in this environment may be more exposed to pollen when playing outdoors or in barns where hay for feeding cattle is kept. High exposure to allergens may thus contribute to the development of tolerance in these children, as has been shown in animal experiments [20]. Repeated exposure of the upper respiratory or gastrointestinal mucosae to microgram levels of protein antigen leads to the preferential suppression of TH2-dependent IgE production via immune deviation. Consequently, it has been suggested to expose children to a mixture comprising the 3–4 dominant inhalant allergens from a particular environment as a measure of primary prevention of allergic respiratory disease [21]. Thus, the possibility exists that living in a agricultural environment provides a model of such primary prevention.

Previous studies have pointed towards a protective role of early childhood exposure to infectious diseases for the development of atopic sensitization and hay fever [22]. Although the number of siblings was adjusted for, a factor which has been postulated to indicate increased childhood exposure to microbial infections [23,24], it is conceivable that the farming environment in itself (e.g. contact to cattle in barns) provides additional exposure to microbial antigens from both commensal organisms and pathogens, thus, stimulating the immune response of farmers' children to a TH1 immunity.

Alternatively, farming as a parental occupation may be an indicator of a more traditional lifestyle which has been proposed to explain the differences in atopic sensitization between Eastern and Western European populations [2,25]. Although the study assessed some of the most important socioeconomic and environmental factors by questionnaire and controlled for these in the analyses, it cannot be ruled out that uncontrolled confounding may still influence the results of this study. For example, dietary factors are potentially important and have not been assessed in the present study. It has recently been postulated that changes in the consumption of omega-3 fatty acids and fresh foods containing antioxidants and magnesium may be responsible for the higher prevalence of asthma observed in affluent countries [26–28]. All these factors may have a potential influence on inflammatory reactions. However, whether dietary factors interfere with the production of specific IgE antibodies against allergens has still to be shown. In addition, dietary factors during pregnancy may be important as the overall health of the mother through pregnancy and, therefore, the growth trajectory of the fetus may have particular relevance [29,30].

In summary, this study indicates that children from a farming population represent an informative population to study the role of environmental factors in the development of atopic sensitization. Whether the observed protective effect of the farming environment on atopic sensitization is explained by high exposure to microbial stimulation in childhood, by different exposures to dietary factors or other indicators of a 'traditional lifestyle', or whether it is the result of preferential suppression of TH2-dependent IgE production due to high allergen exposure remains to be elucidated.

Acknowledgements

SCARPOL team consists of the authors and: I. Gasser (Basel), Ch. E. Minder (Bern), Th. Küenzle (Bern) and A. Peeters (Zürich).

This study was supported by a grant of the Swiss National Research Foundation (#4026–033109). Ch. Braun-Fahrländer was supported by the grant #32–30252.90 from the same agency.

References

1 Brabäck L, Kälvesten L. Urban living as a risk factor for atopic sensitization in Swedish schoolchildren. Pediatr Allergy Immunol 1991; 2:14–9.
2 Björksten B. Risk factors in early childhood for the development of atopic diseases. Allergy 1994; 49:400–7.
3 Popp W, Zwick H, Steyrer K, Rauscher H, Wanke T. Sensitization to aeroallergens depends on environmental factors. Allergy 1989; 44:572–5.
4 Riedel F, Krämer M, Scheibenbogen C, Rieger CHL. Effects of SO_2 exposure on allergic sensitization in the guinea pig. J Allergy Clin Immunol 1988; 82:527–34.
5 Matsumura Y. The effects of ozone, nitrogen dioxide, and sulfur dioxide on the experimentally induced allergic respiratory disorders in guinea pigs. Am Rev Respir Dis 1970; 102:430–7.
6 von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Röll G, Thiemann H-H. Prevalence of asthma and atopy in two areas of West and East Germany. Am J Respir Crit Care Med 1994; 149:358–64.
7 Brabäck L, Breborowicz A, Dreborg S, Knutsson A, Pieklik H, Björkstén B. Atopic sensitization and respiratory symptoms among Polish and Swedish schoolchildren. Clin Exp Allergy 1994; 24:826–35.
8 Braun-Fahrländer Ch, Vuille JC, Sennhauser FH et al. Respiratory health and long term exposure to air pollutants in

Swiss schoolchildren. Am J Respir Crit Care Med 1997; 155:1042–9.
9 Braun-Fahrländer Ch, Wüthrich B, Gassner M et al. Prävalenz und Risikofaktoren einer allergischen Sensibilisierung bei Schulkindern in der Schweiz. Allergologie 1998. In press.
10 Gassner M. Allergie und Umwelt. Allergologie 1989; 12:492–502.
11 Von Mutius E, Illi S, Nicolai T, Martinez F. Relation of indoor heating with asthma, allergic sensitization, and bronchial responsiveness: survey of children in South Bavaria. BMJ 1996; 312:1448–50.
12 Asher MI, Anderson HR, Beasley R et al. International study of asthma and allergies in childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483–91.
13 Braun-Fahrländer Ch, Wüthrich B, Gassner M et al. Validation of a rhinitis symptom questionnaire (ISAAC core questions) in a population of Swiss school children visiting the School Health Services. Pediatr Allergy Immunol 1997; 8:75–82.
14 Kohler F, Kohler Ch, Patris A, Grillat JP. Fréquence de l'allergie pollinique chez les agriculteurs par rapport aux autres catégories socio-professionnelles. Rev Fr Allergol 1983; 23:119–24.
15 Rautalahti M, Terho EO, Vohlonen I, Husman K. Atopic sensitization of dairy farmers to work-related and common allergens. Eur J Respir Dis 1987; 71 (Suppl. 152):155–64.
16 Iversen M, Pedersen B. The prevalence of allergy in Danish farmers. Allergy 1990; 45:347–53.
17 Sigsgaard T, Hjort C, Omland O, Miller MR, Pedersen OF. Skin prick test to house dust mite, asthma and hyperreactivity in a cohort of young rurals. Eur Respir J 1996; 9 (Suppl.):379s.
18 Aberg N. Asthma and allergic rhinitis in Swedish conscripts. Clin Exp Allergy 1989; 19:59–63.
19 Kilpeläinen M, Terho EO, Koskenvuo M. Asthma and atopic diseases among Finish university students. Eur Respir J 1997; 10 (Suppl.):143s.
20 Holt PG. A potential vaccine strategy for asthma and allied atopic diseases during early childhood. Lancet 1994; 344:456–8.
21 Holt PG, Sly PD. Allergic respiratory disease: strategic targets for primary prevention during childhood. Thorax 1997; 52:1–4.
22 Martinez FD. Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? Thorax 1994; 49:1189–91.
23 Strachan DP. Hay fever, hygiene, and household size. BMJ 1989; 299:1259–60.
24 Mutius E, Martinez FD, Fritzsch C, Nicolai T, Reitmair P, Thiemann HH. Skin test reactivity and number of siblings. BMJ 1994; 308:692–5.
25 Wichmann HE. Environment, life-style and allergy: the German answer. Allergo J 1995; 4:315–6.
26 Seaton A, Godden DJ, Brown K. Increase in asthma: a more toxic environment or a more susceptible population. Thorax 1994; 49:171–4.
27 Britton J, Pavord I, Richards K et al. Dietary magnesium, lung function, wheezing, and airway hyperreactivity in a random adult population sample. Lancet 1994; 344:357–62.
28 Black PN, Sharpe S. Dietary fat and asthma: is there a connection. Eur Respir J 1997; 10:6–12.
29 Godfrey KM, Barker DJP, Osmond C. Disproportionate fetal growth and raised IgE concentration in adult life. Clin Exp Allergy 1994; 24:641–8.
30 Fergusson DM, Crane J, Beasley R, Horwood LJ. Perinatal factors and atopic disease in childhood. Clin Exp Allergy 1997; 27:1394–401.

The New England Journal of Medicine

VOLUME 347 SEPTEMBER 19, 2002 NUMBER 12

ENVIRONMENTAL EXPOSURE TO ENDOTOXIN AND ITS RELATION TO ASTHMA IN SCHOOL-AGE CHILDREN

CHARLOTTE BRAUN-FAHRLÄNDER, M.D., JOSEF RIEDLER, M.D., UDO HERZ, PH.D., WALTRAUD EDER, M.D., MARCO WASER, M.SC., LETICIA GRIZE, PH.D., SOYOUN MAISCH, M.D., DAVID CARR, B.SC., FLORIAN GERLACH, ALBRECHT BUFE, M.D., PH.D., ROGER P. LAUENER, M.D., RUDOLF SCHIERL, PH.D., HARALD RENZ, M.D., DENNIS NOWAK, M.D., AND ERIKA VON MUTIUS, M.D., FOR THE ALLERGY AND ENDOTOXIN STUDY TEAM

ABSTRACT

Background In early life, the innate immune system can recognize both viable and nonviable parts of microorganisms. Immune activation may direct the immune response, thus conferring tolerance to allergens such as animal dander or tree and grass pollen.

Methods Parents of children who were 6 to 13 years of age and were living in rural areas of Germany, Austria, or Switzerland where there were both farming and nonfarming households completed a standardized questionnaire on asthma and hay fever. Blood samples were obtained from the children and tested for atopic sensitization; peripheral-blood leukocytes were also harvested from the samples for testing. The levels of endotoxin in the bedding used by these children were examined in relation to clinical findings and to the cytokine-production profiles of peripheral-blood leukocytes that had been stimulated with lipopolysaccharide and staphylococcal enterotoxin B. Complete data were available for 812 children.

Results Endotoxin levels in samples of dust from the child's mattress were inversely related to the occurrence of hay fever, atopic asthma, and atopic sensitization. Nonatopic wheeze was not significantly associated with the endotoxin level. Cytokine production by leukocytes (production of tumor necrosis factor α, interferon-γ, interleukin-10, and interleukin-12) was inversely related to the endotoxin level in the bedding, indicating a marked down-regulation of immune responses in exposed children.

Conclusions A subject's environmental exposure to endotoxin may have a crucial role in the development of tolerance to ubiquitous allergens found in natural environments. (N Engl J Med 2002;347:869-77.)

ASTHMA is the most common chronic disease in childhood and accounts for substantial morbidity and health care costs. Although various environmental factors have been thought to play key parts in the development of asthma and allergies,[1-3] the causes of these diseases remain unclear.

One intriguing hypothesis is that changes in the type and degree of stimulation from the microbial environment associated with improvements in public health and hygiene may increase the predisposition to chronic allergic conditions during childhood.[4] Exposure to microbes can occur in the absence of infection. For example, viable and nonviable parts of microorganisms are found in varying concentrations in many indoor and outdoor environments. These microbial substances are recognized by the innate immune system in the absence of overt infection, and they induce a potent inflammatory response.[5] Therefore, environmental exposure to microbial products may have a crucial role during the maturation of a child's immune response, causing the development of tolerance to other components of his or her natural environment, such as pollen and animal dander.

We investigated the relation between exposure to microbial products and the occurrence of childhood asthma and allergies in an environment rich in op-

From the Institute of Social and Preventive Medicine, Basel, Switzerland (C.B.-F., M.W., L.G.); Children's Hospital Salzburg, Salzburg, Austria (J.R., W.E.); the Department of Clinical Chemistry and Molecular Diagnostics, Hospital of the Philipps University, Marburg, Germany (U.H., H.R.); the Dr. von Hauner Children's Hospital, Munich, Germany (S.M., D.C., F.G., E.M.); the Department of Experimental Pneumology, Ruhr University, Bochum, Germany (A.B.); University Children's Hospital, Zurich, Switzerland (R.P.L.); and the Institute of Occupational and Environmental Medicine, University of Munich, Munich, Germany (R.S., D.N.). Address reprint requests to Dr. Braun-Fahrländer at the Institute of Social and Preventive Medicine, University of Basel, Steinengraben 49, CH-4051 Basel, Switzerland, or at c.braun@unibas.ch.

portunities for such exposure — that is, a rural environment where some families engage in farming. We measured endotoxin — a cell-wall component of gram-negative bacteria — in samples of dust from the mattresses of children and then related the levels of endotoxin to the prevalence of asthma and allergies and to serum levels of specific IgE. We also assessed the cytokine-production profile of peripheral-blood leukocytes after activation of the innate immune system by stimulation with lipopolysaccharide and staphylococcal enterotoxin B.

METHODS

Study Population

This cross-sectional survey was conducted in rural areas of Austria, Germany, and Switzerland, as previously described.[6] Participating parents (2618 of 3504 potential participants [74.7 percent]) were asked to consent to the measurement of specific IgE in their children's serum, the assessment of the cytokine-production profile of the children's peripheral-blood leukocytes after stimulation with lipopolysaccharide and staphylococcal enterotoxin B, and the collection of dust samples from the children's bedding. The final analysis was restricted to 812 children with complete data and similar ethnic origin (categorized as German, Austrian, or Swiss nationality), in order to avoid potential confounding by ethnic background.[7]

Approval to conduct the survey was obtained from the three local ethics committees for human studies and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children.

Dust Sampling

We collected dust by vacuuming each mattress for two minutes per square meter of surface area. The material obtained was divided in two for measurement of endotoxin and allergen content. Dust was collected on special filters provided by the Allergologisk Laboratorium Kopenhagen.[8] All field workers were centrally trained and certified to ensure similarity of sampling.

Measurements of Endotoxin Levels

One dust sample was stored at room temperature and shipped within one week after collection to the central laboratory (in Munich, Germany). Endotoxin content was measured by a kinetic limulus assay, as described by Hollander et al.[9] Endotoxin results were expressed as endotoxin units per milligram of dust and as endotoxin units per square meter of surface area of the sampled mattress. All endotoxin levels were within the limits of detection of the assay.

Measurements of Allergen Levels in Dust Samples

The second dust sample was frozen at −20°C for at least two days and then shipped to one central laboratory (University Children's Hospital Charité, Berlin, Germany) and stored at 4°C until it was analyzed for *Dermatophagoides pteronyssinus* (Der p1), *D. farinae* (Der f1), and *Felis domesticus* (Fel d1), as previously described.[3] The lower limit of detection was 10 ng per gram of dust for Der p1 and Der f1 and 16 ng per gram of dust for Fel d1; results are expressed in nanograms of major allergen per gram of mattress dust. For allergen levels below the limit of detection (9.7 percent for Der p1, 5.5 percent for Der f1, and 0.2 percent for Fel d1), the mean value between zero and the limit of detection was used.

Questionnaire and Interview

The prevalence of diseases and symptoms and potential explanatory and confounding factors were assessed by a questionnaire given to the parents that included the questions of the International Study of Asthma and Allergies in Childhood,[10] as described previously.[6] Farmers' children were defined as children whose parents answered "yes" to the question "Does your child live on a farm?" In an interview with the parents as part of the home visit, we obtained details of the timing of the child's exposure to stables and to farm milk. Exposure to farming during the first year of life was defined as exposure to stables during the first year of life, consumption of milk directly from the farm during the first year of life, or both.

Testing for Specific IgE in Serum

The level of specific IgE against airborne allergens in all serum samples was measured by fluorescence enzyme immunoassay in a central laboratory (University Children's Hospital Charité, Berlin). Atopy was defined by at least one positive test for specific IgE indicating a titer of at least 3.5 kU per liter for one or more of the six airborne allergens (house dust mites, storage mites, grass pollen, birch pollen, cat dander, and cow epithelium).

Assessment of Cytokine Production by Peripheral-Blood Leukocytes

Venous blood was drawn at school from all 812 children. Heparinized blood was diluted in a ratio of 1:8 in RPMI culture medium supplemented with 10 percent heat-inactivated fetal-calf serum to a final volume of 1 ml. Cells were stimulated either with 10 μg of lipopolysaccharide per milliliter for 24 hours or with staphylococcal enterotoxin B for 72 hours at 37°C, in an environment of 5 percent carbon dioxide in humidified air. Cell-free supernatants were stored at −80°C and shipped to the central laboratory for measurement of interferon-γ (limit of detection, 16 pg per milliliter), tumor necrosis factor α (limit of detection, 16 pg per milliliter), interleukin-10 (limit of detection, 8 pg per milliliter), and interleukin-12 (limit of detection, 8 pg per milliliter) by commercially available enzyme-linked immunosorbent assays (OptEIA, Pharmingen). Each sample was tested in duplicate by the serial dilution of a standard supplied by the company with a known cytokine level. Differential blood counts were also performed, and cytokine production was expressed in picograms per 1 million peripheral-blood leukocytes. To ensure consistent performance in sampling and culture procedures, laboratory personnel in the study centers participated in a one-week training and certification program.

Statistical Analysis

Endotoxin levels were $\log_{10}$-transformed. Multivariate logistic-regression analyses, in which the endotoxin level was treated as a continuous variable, were performed with SAS software,[11] with adjustment for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of older siblings (the basic model). In addition, potential confounding by farming status, exposure to farming during the first year of life, exposure to cats or dogs during the first year of life, and allergen levels (log-transformed values for Der f1, Der p1, and Fel d1) was evaluated. We included an interaction term to assess whether the effect of endotoxin on asthma and wheeze in children with atopic sensitization (a specific IgE level of at least 0.35 kU per liter) would be different from the effect in children without atopic sensitization.

To evaluate potential threshold values or other nonlinearity in the relation between exposure and response, S-Plus software was used to perform local nonparametric smoothing.[12] The logit of the rates of symptoms was expressed as a continuous function of endotoxin level, obtained by local nonparametric smoothing with control for the covariates mentioned above. The smoothing parameter for each model was determined on the basis of Akaike's information criterion.[12] In the same way, the association between endotoxin levels and cytokine response was assessed. Cytokine levels were log-transformed, and the association of these levels with the level of endo-

toxin exposure was expressed as the ratio of the covariate-adjusted geometric mean cytokine level in children in the highest quartile of endotoxin exposure to the mean level in children in the lowest quartile. The regression analyses were repeated with a restricted sample of children from nonfarming households with adjustment for known allergy-avoidance measures (removal of pets or carpets because of allergies in the family), exposure to cats or dogs during the first year of life, and exposure to farming during the first year of life.

RESULTS

Complete data were available for 812 children, 319 from farming families and 493 from nonfarming families. The mean (±SD) age was 9.5±1.2 years. The adjusted odds ratios for asthma and hay-fever symptoms in relation to the farming status did not differ significantly between the group with complete data and the group with only the self-administered questionnaire (0.59 vs. 0.48 for asthma and 0.44 vs. 0.32 for hay-fever symptoms).[6] The relations between farming status and environmental-exposure variables and health outcomes are shown in Table 1.

The results of multivariate logistic-regression analyses estimating the effect of the mattress endotoxin level and the endotoxin load on the rates of symptoms and disease, with adjustment for known covariates, are shown in Table 2. The data are presented as adjusted odds ratios for symptoms or disease with an increase from the lowest quartile to the highest quartile of endotoxin exposure. Current endotoxin exposure showed a strong inverse association with hay fever, hay-fever symptoms, and atopic sensitization. Smoothed plots of the prevalence of hay fever, hay-fever symptoms, and atopic sensitization in relation to the level of endotoxin exposure, with control for covariates, showed a largely monotonic decrease in prevalence with an increasing endotoxin load (Fig. 1). Similar results were obtained in analyses in which the endotoxin level was used as the exposure variable (data not shown).

An inverse relation was also found between the level of endotoxin exposure and the capacity of peripheral-blood leukocytes to produce inflammatory and regulatory cytokines after stimulation with lipopolysaccharide (Fig. 2). The associations between endotoxin exposure (in endotoxin units per square meter) and the production of tumor necrosis factor α, interferon-γ, interleukin-10, and interleukin-12, expressed

TABLE 1. ENVIRONMENTAL EXPOSURE AND PREVALENCE OF HEALTH OUTCOMES, ACCORDING TO FARMING STATUS.*

VARIABLE	CHILDREN FROM FARMING HOUSEHOLDS (N=319)	CHILDREN FROM NONFARMING HOUSEHOLDS (N=493)	P VALUE
	geometric mean exposure (5th–95th percentile)		
Environmental exposure			
Endotoxin level (units/mg of dust)	37.8 (14.4–88.9)	22.8 (8.2–62.9)	<0.001
Endotoxin load (units/m² of mattress surface area)	29,897 (5452–157,208)	14,456 (2915–75,730)	<0.001
Der f1 (ng/g of dust)	528.7 (5–51,990)	610.3 (5–54,160)	0.54
Der p1 (ng/g of dust)	7,092.4 (133–104,110)	1,417.1 (5–104,060)	<0.001
Fel d1 (ng/g of dust)	5,405.6 (356–144,600)	5,744.1 (204–434,460)	0.69
	no. (% [95% CI])		
Health outcomes			
Hay fever	13 (4.1 [1.9–6.2])	52 (10.5 [7.8–13.5])	<0.001
Sneezing and itchy eyes during previous yr	19 (6.0 [3.3–8.7])	62 (12.6 [9.7–16.0])	0.002
Atopic sensitization	55 (17.2 [13.1–21.4])	116 (23.5 [19.8–27.3])	0.03
Atopic asthma	10 (3.1 [1.2–5.0])	29 (5.9 [3.8–8.0])	0.07
Nonatopic asthma	5 (1.6 [0.2–2.9])	13 (2.6 [1.2–5.0])	0.31
Atopic wheeze	15 (4.7 [2.4–7.0])	29 (5.9 [3.8–8.0])	0.47
Nonatopic wheeze	5 (1.6 [0.2–2.9])	30 (6.1 [4.0–8.2])	0.002

*Children were considered to have hay fever if their parents reported a physician's diagnosis of hay fever; to have had sneezing and itchy eyes (symptoms of hay fever) during the previous year if their parents gave a positive response to a question about these symptoms; to have atopic sensitization if they had a specific IgE titer of at least 3.5 kU per liter; to have atopic asthma if their parents reported a physician's diagnosis of asthma or if they had recurrent asthmatic obstruction of the airway or spastic bronchitis and a specific IgE titer of at least 0.35 kU per liter; to have nonatopic asthma if their parents reported a physician's diagnosis of asthma or if they had recurrent asthmatic obstruction of the airway or spastic bronchitis and a specific IgE titer of less than 0.35 kU per liter; to have atopic wheeze if their parents reported that they had had wheezing or whistling in the chest during the previous 12 months and they had a specific IgE titer of at least 0.35 kU per liter; and to have nonatopic wheeze if their parents reported that they had had wheezing or whistling in the chest during the previous 12 months and they had a specific IgE titer of less than 0.35 kU per liter. CI denotes confidence interval, Der f1 *Dermatophagoides farinae*, Der p1 *D. pteronyssinus*, and Fel d1 *Felis domesticus*.

TABLE 2. ASSOCIATIONS BETWEEN CURRENT ENDOTOXIN EXPOSURE (LEVEL AND LOAD) AND ASTHMA, WHEEZE, HAY FEVER, AND ATOPIC SENSITIZATION IN THE TOTAL SAMPLE AND IN THE SUBGROUP OF CHILDREN FROM NONFARMING HOUSEHOLDS.

HEALTH OUTCOME	TOTAL SAMPLE (N=812)		CHILDREN FROM NONFARMING HOUSEHOLDS (N=493)	
	ENDOTOXIN LEVEL	ENDOTOXIN LOAD	ENDOTOXIN LEVEL	ENDOTOXIN LOAD
	adjusted odds ratio (95% CI)*			
Hay fever	0.58 (0.39–0.85)†	0.53 (0.35–0.81)†	0.79 (0.52–1.19)	0.56 (0.33–0.95)†
Sneezing and itchy eyes during previous yr	0.61 (0.43–0.86)†	0.50 (0.34–0.72)†	0.70 (0.47–1.05)	0.46 (0.28–0.76)†
Atopic sensitization‡	0.78 (0.60–1.01)	0.76 (0.58–0.98)†	0.80 (0.59–1.08)	0.73 (0.51–1.04)
Atopic asthma	0.73 (0.44–1.19)	0.48 (0.28–0.81)†	0.68 (0.39–1.19)	0.52 (0.25–1.07)
Nonatopic asthma	1.25 (0.62–2.51)	1.13 (0.57–2.26)	1.29 (0.62–2.68)	1.00 (0.46–2.21)
Atopic wheeze	0.89 (0.57–1.39)	0.62 (0.39–0.99)†	0.79 (0.46–1.33)	0.64 (0.33–1.25)
Nonatopic wheeze	0.97 (0.58–1.61)	1.14 (0.68–1.90)	1.36 (0.86–2.14)	1.82 (1.04–3.18)†

*Odds ratios are for the occurrence of the given symptom or disease with an increase in the endotoxin measure from the lowest quartile to the highest quartile; analyses were adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, and number of older siblings. The analysis of the subgroup of children from nonfarming households was also adjusted for allergen-avoidance measures, exposure to pets during the first year of life, exposure to stables during the first year of life, and consumption of milk directly from a farm during the first year of life.

†$P \leq 0.05$ for the comparison between children in the lowest quartile of endotoxin exposure and children in the highest quartile.

‡Atopic sensitization was defined by a specific IgE titer of at least 3.5 kU per liter.

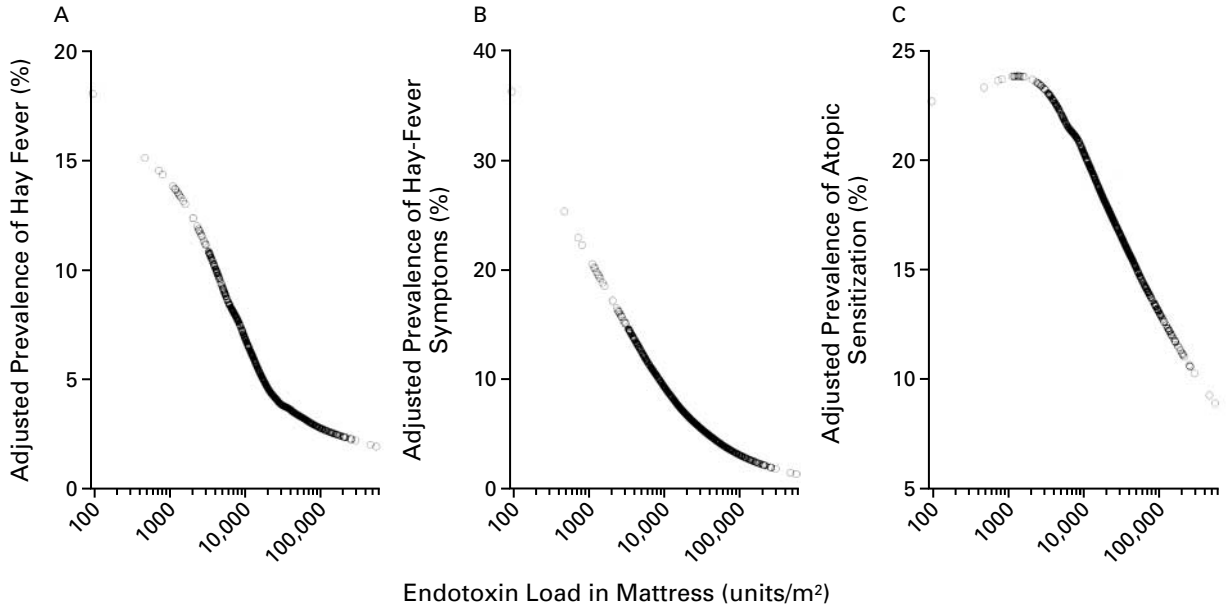


Figure 1. Smoothed Plots of the Prevalence of Hay Fever (Panel A), Hay-Fever Symptoms (Panel B), and Atopic Sensitization (Panel C) in Relation to the Log-Transformed Endotoxin-Load Values.

The analyses controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings. For each outcome, there was a monotonic decrease with increasing endotoxin load. A smoothing span of 0.9 was used for all three graphs.

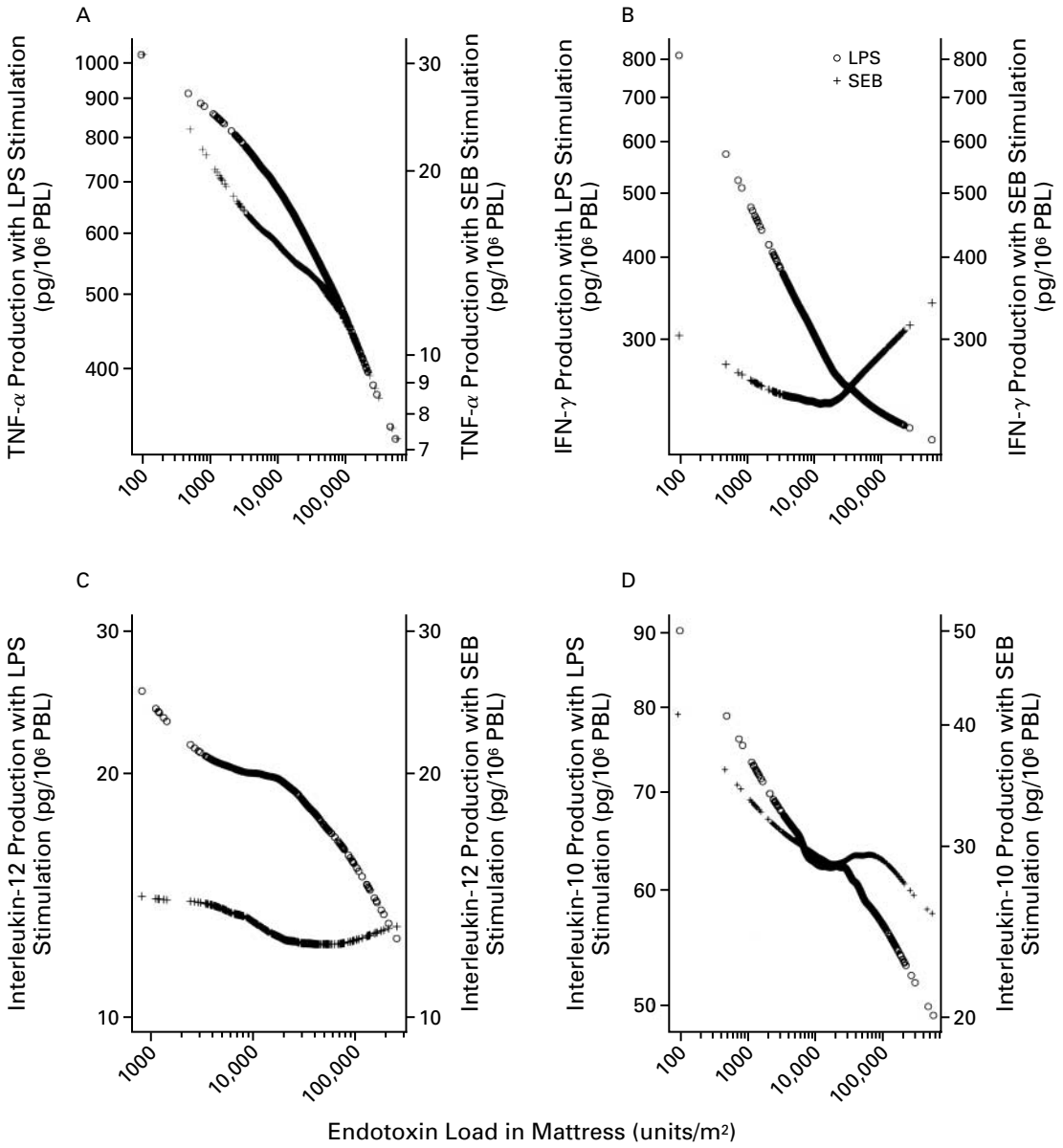


Figure 2. Smoothed Plots of the Log-Transformed Capacity of Peripheral-Blood Leukocytes (PBL) to Produce Tumor Necrosis Factor α (TNF-α) (Panel A), Interferon-γ (IFN-γ) (Panel B), Interleukin-12 (Panel C), and Interleukin-10 (Panel D) after Stimulation with Lipopolysaccharide (LPS) or Staphylococcal Enterotoxin B (SEB) in Relation to the Log-Transformed Endotoxin-Load Values.

Analyses were controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings; the analysis shows an inverse relation between the level of endotoxin exposure and cytokine response, except in the case of the production of IFN-γ after SEB stimulation. A smoothing span of 0.9 was used for all four graphs.

as ratios of the mean level of cytokine production for children in the highest quartile of endotoxin exposure to the mean level for children in the lowest quartile, were 0.81 (95 percent confidence interval, 0.74 to 0.89), 0.80 (95 percent confidence interval, 0.70 to 0.92), 0.93 (95 percent confidence interval, 0.81 to 1.07), and 0.87 (95 percent confidence interval, 0.77 to 0.98), respectively. The corresponding results after stimulation with staphylococcal enterotoxin B were 0.83 (95 percent confidence interval, 0.74 to 0.93), 1.05 (95 percent confidence interval, 0.95 to 1.17), 0.97 (95 percent confidence interval, 0.84 to 1.11), and 0.96 (95 percent confidence interval, 0.86 to 1.06), respectively.

The association between endotoxin exposure and wheeze during the past year showed a different exposure–response pattern. There was a strong negative association for atopic wheeze and asthma, whereas for nonatopic wheeze and asthma, there was a nonsignificant trend toward increasing prevalence with increases in the current level of endotoxin exposure (Table 2 and Fig. 3). However, the term for the interaction between the level of endotoxin exposure and atopic status did not reach statistical significance. Exposure to farming in the first year of life showed a strong inverse association with all health outcomes, including nonatopic wheeze and asthma, independently of the current level of endotoxin exposure (Table 3). Additional adjustment for other potential confounders, including the levels of allergens (Der f1, Der p1, and Fel d1) in mattress dust, farming status, exposure to pets during the first year of life, and exposure to farming during the first year of life, did not change the results. To evaluate whether the results might be generalized to a nonfarming population and to adjust for potential uncontrolled confounding associated with a farming lifestyle, we restricted the sample to children from nonfarming households and also adjusted for exposure to stables and consumption of milk directly from the farm during the first year of life. Again, strong negative associations — albeit not all statistically significant (probably because of the sample size) — between the level of endotoxin exposure and atopic outcomes were observed, whereas positive associations were found for nonatopic wheeze (Table 2).

DISCUSSION

These findings suggest that environmental exposure to microbial products, as measured by the endotoxin levels in mattress dust, is associated with a significant decrease in the risk of hay fever, atopic sensitization, atopic asthma, and atopic wheeze in child-

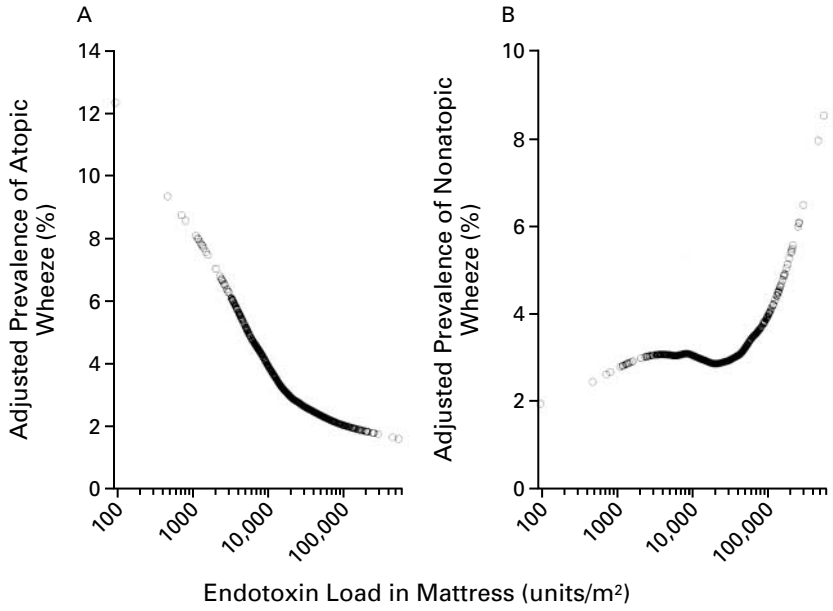


Figure 3. Smoothed Plots of the Prevalence of Atopic Wheeze (Panel A) and Nonatopic Wheeze (Panel B) in Relation to the Log-Transformed Endotoxin-Load Values.

The analyses were controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings. There was a negative association for atopic wheeze, whereas for nonatopic wheeze, there was a nonsignificant positive trend with increasing levels of current endotoxin exposure. For Panel A, a smoothing span of 0.9 was used; for Panel B, a span of 0.5 was used.

TABLE 3. ASSOCIATION OF THE PREVALENCE OF SYMPTOMS AND DISEASE WITH THE CURRENT ENDOTOXIN LOAD AND EXPOSURE TO FARMING DURING THE FIRST YEAR OF LIFE.*

HEALTH OUTCOME	EXPOSURE TO FARMING DURING THE FIRST YEAR†	CURRENT ENDOTOXIN EXPOSURE‡
	odds ratio (95% CI)	
Hay fever	0.26 (0.13–0.52)§	0.61 (0.40–0.95)§
Sneezing and itchy eyes after 1 yr of age	0.55 (0.31–0.97)§	0.53 (0.36–0.77)§
Atopic sensitization¶	0.45 (0.30–0.68)§	0.83 (0.63–1.09)
Atopic asthma	0.42 (0.18–0.96)§	0.52 (0.30–0.90)§
Nonatopic asthma	0.48 (0.16–1.41)	1.22 (0.60–2.46)
Atopic wheeze	0.59 (0.28–1.23)	0.66 (0.41–1.07)
Nonatopic wheeze	0.43 (0.19–0.97)§	1.23 (0.73–2.06)

*Odds ratios are for the occurrence of the given symptom or disease with an increase in exposure to farming or the endotoxin load from the lowest quartile to the highest quartile. Analyses were adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, and number of older siblings.

†Odds ratios were also adjusted for current endotoxin exposure.

‡Odds ratios were also adjusted for exposure to farming during the first year of life.

§$P \leq 0.05$ for the comparison between children in the lowest quartile of the exposure variable and those in the highest quartile.

¶Atopic sensitization was defined by a specific IgE titer of at least 3.5 kU per liter.

hood. This protective effect was observed in children from farming and nonfarming households, indicating that even the lower levels of exposure that occur in nonfarming environments may favorably influence the risk of atopic diseases in childhood.

The mechanisms by which endotoxin exposure may protect against the development of atopic immune responses and diseases are not fully understood. Our findings suggest that by the time a child reaches school age, high levels of environmental exposure to endotoxin have resulted in a marked suppression of the capacity for cytokine production in response to activation of the innate immune system. Whereas lipopolysaccharide stimulation triggers an innate immune response by activating mainly antigen-presenting cells, staphylococcal enterotoxin B also activates T cells, resulting in a somewhat different pattern of cytokine production. Reduced responsiveness to stimulation with lipopolysaccharide after previous stimulation with lipopolysaccharide is a phenomenon referred to in the literature as lipopolysaccharide tolerance.[13,14] Our results suggest that such a down-regulation occurs in vivo as a consequence of long-term exposure to environmental endotoxin. Whether this down-regulation is merely a biologic marker of the exposure or is causally related to the decreased rate of atopy cannot be determined on the basis of our data; it is an area in which further exploration is needed. It has been suggested that the innate immune response has an instructive role in adaptive immunity.[15] Differential expression of lipopolysaccharide receptors in children from farming and nonfarming households has recently been reported,[16] suggesting that the innate immune system responds to the high microbial burden of the farming environment.

Although only current endotoxin exposure was measured, the levels are likely to reflect long-term exposure. Therefore, long-term, high-level environmental exposure may favor a state of tolerance,[14] which may prevent the development of allergic immune responses. We demonstrated that exposure during the first year of life to stables and other aspects of farm life that are likely to reflect exposure to microbial products has a strong protective effect against the occurrence of asthma and atopy at school age. However, independent of and in addition to this effect, endotoxin exposure at school age was associated with a markedly decreased risk of atopic outcomes. This protective effect was also seen in children with no exposure to farming whose mattress endotoxin levels were similar to levels found in urban homes in the Netherlands[17] and urban areas in the United States,[18,19] suggesting that exposure to ubiquitous microbial products strongly affects the development of atopy and childhood asthma. The increase in the frequency of asthma in inner-city areas of the United States, by contrast, may be related to other types of environmental exposure.

The protective effect of endotoxin exposure at school age was observed only for atopic wheeze and asthma, not for nonatopic wheeze. Childhood asthma is a complex syndrome with multiple illnesses involving wheezing that develop during the infant, toddler, school-age, and adolescent years, as has been shown in several long-term, prospective surveys in which children were followed from birth to adolescence and adulthood.[20-22] Although, in many cases, asthma is associated with atopic sensitization to a variety of allergens, illnesses involving wheezing also occur in the absence of increased IgE responses. Variations in genetic background, environmental factors, and the interplay among them are likely to account for the varying clinical presentations of wheeze. In studies of human exposure[23] and in studies of animals,[24] endotoxin has been shown to induce airway hyperresponsiveness in healthy, nonatopic subjects but to decrease airway responsiveness in sensitized animals, supporting the notion that the effect is modified by atopy, as we observed. In our study, exposure to farming in the first year of life had a protective effect against nonatopic wheeze, whereas exposure to endotoxin at school age was related to an increased risk. Therefore, not only an exposed subject's atopic status but also the timing of the exposure determines its beneficial or detrimental effects.

Endotoxin was measured in mattress dust, since children come into close contact with the microbial environment of their beds while sleeping and since the reproducibility of repeated endotoxin measurements is greater for dust from the bed than for dust from the floor.[25] Endotoxin measurements in dust from the bed have been reported to show little variation over time, with nonsignificant differences over a six-month period.[19] Environmental endotoxin levels are therefore likely to reflect longer-term exposure to microbial compounds. However, the cross-sectional design of our study limited our ability to determine precisely the duration of exposure represented by current endotoxin measurements, and prospective studies are clearly needed. We did not assess other bacterial components, such as nonmethylated cytidine phosphate guanosine dinucleotides specific for prokaryotic DNA (CpG motifs) or cell-wall components from atypical mycobacteria or gram-positive bacteria such as lipoteichoic acid, which are known to affect immune responses in ways similar to that of endotoxin.[26,27] The observed protective effect associated with endotoxin levels in mattress dust is therefore likely to reflect the effect of exposure to a much broader spectrum of microbial compounds than gram-negative bacteria alone.

The results of our study indicate that environmental exposure to microbial products as assessed by the measurement of endotoxin levels in mattress dust is associated with the development of tolerance toward ubiquitous allergens found in natural environments. Mechanisms relating to the recognition of these microbial compounds by the innate immune system and the regulation of the resulting inflammatory responses through adaptive immunity are likely to be of key importance for the development of atopic illnesses such as hay fever and childhood asthma and wheeze. These insights may foster the generation of novel strategies aimed at the prevention of these diseases.

Supported by a grant from the Health Department of the Government of Salzburg, Austria; by the Zurich Lung Association; by a grant from the United Bank of Switzerland; by a grant from the Swiss National Science Foundation; and by the Bavarian Ministry for the Environment.

We are indebted to our collaborators on the Allergy and Endotoxin Study Team — Otto Holst (Borstel, Germany), Mynda Schreuer and Gerd Oberfeld (Salzburg, Austria), and Felix Sennhauser (Zurich, Switzerland); to Dr. Susan Lau and her team, University Children's Hospital Charité, Berlin, for the determination of the allergen level in dust samples and the measurement of specific IgE levels in serum; and to Angelika Kronseder and Stefan Gröbmair, Institute of Occupational and Environmental Medicine, University of Munich, for the endotoxin measurements.

REFERENCES

1. D'Amato G, Liccardi G, D'Amato M. Environmental risk factors (outdoor air pollution and climatic changes) and increased trend of respiratory allergy. J Investig Allergol Clin Immunol 2000;10:123-8.
2. Gold DR. Environmental tobacco smoke, indoor allergens, and childhood asthma. Environ Health Perspect 2000;108:Suppl 4:643-51.
3. Wahn U, Lau S, Bergmann R, et al. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 1997;99:763-9.
4. Strachan DP. Family size, infection and atopy: the first decade of the "hygiene hypothesis." Thorax 2000;55:Suppl 1:S2-S10.
5. Delves PJ, Roitt IM. The immune system. N Engl J Med 2000;343:37-49.
6. Riedler J, Braun-Fahrländer C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-33.
7. Kabesch M, Schaal W, Nicolai T, von Mutius E. Lower prevalence of asthma and atopy in Turkish children living in Germany. Eur Respir J 1999; 13:577-82.
8. Phase II modules of the International Study of Asthma and Allergies in Childhood (ISAAC). Münster, Germany: ISAAC, 1998.
9. Hollander A, Heederik D, Versloot P, Douwes J. Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. Am Ind Hyg Assoc J 1993;54:647-53.
10. Asher MI, Keil U, Anderson HR, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995;8:483-91.
11. SAS, release 8.02. Cary, N.C.: SAS Institute, 2001.
12. S-PLUS. Seattle: MathSoft, 2000.
13. West MA, Heagy W. Endotoxin tolerance: a review. Crit Care Med 2002;30:Suppl:S64-S73.
14. Schade U, Schlegel J, Hofmann K, Brade H, Flach R. Endotoxin tolerant mice produce an inhibitor of tumor necrosis factor synthesis. J Endotoxin Res 1996;3:455-62.
15. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science 1996;272:50-3.
16. Lauener R, Birchler T, Adamski J, et al. Expression of CD14 and toll-like receptor 2 in farmers' and non-farmers' children. Lancet 2002;360: 465-6.
17. Douwes J, Zuidhof A, Doekes G, et al. (1→3)-Beta-D-glucan and endotoxin in house dust and peak flow variability in children. Am J Respir Crit Care Med 2000;162:1348-54.
18. Park JH, Gold DR, Spiegelman DL, Burge HA, Milton DK. House dust endotoxin and wheeze in the first year of life. Am J Respir Crit Care Med 2001;163:322-8.

19. Gereda JE, Leung DY, Thatayatikom A, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 2000;355:1680-3.
20. Strachan DP, Butland BK, Anderson HR. Incidence and prognosis of asthma and wheezing illness from early childhood to age 33 in a national British cohort. BMJ 1996;312:1195-9.
21. Silverman M, Wilson N. Wheezing phenotypes in childhood. Thorax 1997;52:936-7.
22. Martinez FD, Wright AL, Taussig LM, et al. Asthma and wheezing in the first six years of life. N Engl J Med 1995;332:133-8.
23. Rylander R, Bake B, Fischer JJ, Helander IM. Pulmonary function and symptoms after inhalation of endotoxin. Am Rev Respir Dis 1989;140: 981-6.
24. Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 2000;22:604-12.
25. Park JH, Spiegelman DL, Burge HA, Gold DR, Chew GL, Milton DK. Longitudinal study of dust and airborne endotoxin in the home. Environ Health Perspect 2000;108:1023-8.
26. Abou-Zeid C, Gares MP, Inwald J, et al. Induction of a type 1 immune response to a recombinant antigen from Mycobacterium tuberculosis in Mycobacterium vaccae. Infect Immun 1997;65:1856-62.
27. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996;64:1906-12.

22. Weiner BH, Ockene IS, Levine PH, et al. Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. N Engl J Med 1986; 315:841-6.
23. Davis HR, Bridenstine RT, Vesselinovitch D, Wissler RW. Fish oil inhibits development of atherosclerosis in rhesus monkeys. Arteriosclerosis 1987; 7:441-9.
24. Dinarello CA, Lonnemann G, Maxwell R, Shaldon S. Ultrafiltration to reject human interleukin-1-inducing substances derived from bacterial cultures. J Clin Microbiol 1987; 25:1233-8.
25. Lisi PJ, Chu CW, Koch GA, Endres S, Lonnemann G, Dinarello CA. Development and use of a radioimmunoassay for human interleukin-1 beta. Lymphokine Res 1987; 6:229-44.
26. van der Meer JW, Endres S, Lonnemann G, et al. Concentrations of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells in vitro. J Leukocyte Biol 1988; 43:216-23.
27. Lonnemann G, Endres S, van der Meer JW, Cannon JG, Dinarello CA. A radioimmunoassay for human interleukin-1α: measurement of IL-1α produced in vitro by human blood mononuclear cells stimulated with endotoxin. Lymphokine Res 1988; 7:75-84.
28. Georgilis K, Schaefer C, Dinarello CA, Klempner MS. Human recombinant interleukin 1 beta has no effect on intracellular calcium or on functional responses of human neutrophils. J Immunol 1987; 138:3403-7.
29. Siguel EN, Chee KM, Gong JX, Schaefer EJ. Criteria for essential fatty acid deficiency in plasma as assessed by capillary column gas-liquid chromatography. Clin Chem 1987; 33:1869-73.
30. von Schacky C, Fischer S, Weber PC. Long-term effect of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. J Clin Invest 1985; 76:1626-31.
31. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR. Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. N Engl J Med 1985; 312:1210-6.
32. Cuturi MC, Murphy M, Costa-Giomi MP, Weinmann R, Perussia B, Trinchieri G. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med 1987; 165:1581-94.
33. Matsushima K, Procopio A, Abe H, Scala G, Ortaldo JR, Oppenheim JJ. Production of interleukin 1 activity by normal human peripheral blood B lymphocytes. J Immunol 1985; 135:1132-6.
34. Scala G, Allavena P, Djeu JY, et al. Human large granular lymphocytes are potent producers of interleukin-1. Nature 1984; 309:56-9.
35. Lovett DH, Sterzel RB, Ryan JL, Atkins E. Production of an endogenous pyrogen by glomerular mesangial cells. J Immunol 1985; 134:670-2.
36. Libby P, Ordovas JM, Auger KR, Robbins AH, Birinyi LK, Dinarello CA. Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. Am J Pathol 1986; 124:179-85.
37. Sauder DN. Biologic properties of epidermal cell thymocyte-activating factor (ETAF). J Invest Dermatol 1985; 85:Suppl:176s-179s.
38. van Furth R, Raeburn JA, van Zwet TL. Characteristics of human mononuclear phagocytes. Blood 1979; 54:485-500.
39. Kunkel SL, Chensue SW, Phan SH. Prostaglandins as endogenous mediators of interleukin 1 production. J Immunol 1986; 136:186-92.
40. Knudsen PJ, Dinarello CA, Strom TB. Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. J Immunol 1986; 137:3189-94.
41. Rola-Pleszczynski M, Lemaire I. Leukotrienes augment interleukin 1 production by human monocytes. J Immunol 1985; 135:3958-61.
42. Dinarello CA, Bishai I, Rosenwasser LJ, Coccani F. The influence of lipoxygenase inhibitors on the in vitro production of human leukocytic pyrogen and lymphocyte activating factor (interleukin-1). Int J Immunopharmacol 1984; 6:43-50.
43. Kunkel SL, Chensue SW. Arachidonic acid metabolites regulate interleukin-1 production. Biochem Biophys Res Commun 1985; 128:892-7.

ASSOCIATION OF ASTHMA WITH SERUM IgE LEVELS AND SKIN-TEST REACTIVITY TO ALLERGENS

BENJAMIN BURROWS, M.D., FERNANDO D. MARTINEZ, M.D., MARILYN HALONEN, PH.D., ROBERT A. BARBEE, M.D., AND MARTHA G. CLINE, M.S.

Abstract We investigated the association of self-reported asthma or allergic rhinitis with serum IgE levels and skin-test reactivity to allergens in 2657 subjects in a general-population study. Regardless of the subjects' status with respect to atopy or their age group, the prevalence of asthma was closely related to the serum IgE level standardized for age and sex ($P<0.0001$), and no asthma was present in the 177 subjects with the lowest IgE levels for their age and sex (>1.46 SD below the mean). The log odds ratio increased linearly with the serum IgE level after we controlled for possible confounders and the degree of reactivity to skin tests. In contrast, allergic rhinitis appeared to be associated primarily with skin-test reactions to common aeroallergens, independently of the serum IgE level.

We conclude that asthma is almost always associated with some type of IgE-related reaction and therefore has an allergic basis, although not all the allergic stimuli that cause asthma appear to have been included in the battery of common aeroallergens we used to assess atopic status. These findings challenge the concept that there are basic differences between so-called allergic ("extrinsic") and nonallergic ("intrinsic") forms of asthma. (N Engl J Med 1989; 320:271-7.)

IT is generally accepted that there are both allergic and nonallergic forms of asthma, and the distinction is usually based on the presence or absence of allergy skin-test reactivity to one or more aeroallergens considered capable of inducing the disease. Since Rackemann introduced the terms in 1918,[1] asthma that can be shown to be related to hypersensitivity to a foreign substance has been described as "extrinsic"; otherwise, the disease has been classified as "intrinsic."

After Ishizaka[2] and Johansson[3] and their colleagues identified immunoglobulin E (IgE) as the antibody responsible for the immediate type of immune response, a number of authors noted marked elevations of IgE in young patients with asthma,[4,5] and some have attempted to distinguish extrinsic from intrinsic disease on the basis of the presence or absence of an elevated serum concentration of IgE.[6] Regardless of the specific criteria, however, extrinsic asthma appears to be the predominant form of the disease among children and young adults, and intrinsic asthma is considered to be more common among older subjects.

These widely accepted concepts, based largely on clinical observations, fail to take into account the relations of both allergy skin-test reactivity (hereafter called atopy) and serum IgE levels to age in the general population. Both tend to decrease with advancing

From the Division of Respiratory Sciences (Westend Laboratories), University of Arizona College of Medicine, Tucson, Ariz. Address reprint requests to Dr. Burrows at the Division of Respiratory Sciences, University of Arizona College of Medicine, Tucson, AZ 85724.

Supported by a Specialized Center of Research Grant (HL-14136) from the National Heart, Lung, and Blood Institute.

age. Although serum IgE reaches its peak level during childhood, atopy continues to develop up to the third decade.[7-12] In addition, serum IgE levels have been shown to be affected by sex and by smoking habits,[13,14] and there is no agreement about the level of serum IgE that exceeds the normal limit.

In the present report, we consider the relation of reported "asthma" to both atopy and the serum IgE level, taking into account their close interrelation as well as their variations with age and sex. Findings for asthma are contrasted with those for reported "allergic rhinitis" among 2657 subjects 6 to 95 years old who were enrolled in an epidemiologic study of a sample from the general population.

Methods

The details of the methods of selection and study of the population have been previously reported.[15] The subjects were selected from a random stratified cluster sample of white non-Mexican-American households in Tucson, Arizona, in 1971. The sampling frame consisted of square blocks stratified on the basis of ethnic group, age of the head of the household, and socioeconomic status. The members of one of each six households in these square blocks were asked to participate. Blood samples were obtained from the subjects at least six years old, and serum IgE levels were measured. The present analyses were limited to the 2657 subjects in whom serum for measurement of IgE had been obtained and allergy skin tests had been performed at the time of enrollment. The average age (±SD) was 44.1±21.9 years, and 54.5 percent of the sample were female subjects. Nonsmokers, exsmokers, and current smokers made up 48.5, 21.5, and 30.0 percent, respectively, of the study group.

The presence of disease was determined by responses to a standardized self-completed questionnaire. "Allergic rhinitis," hereafter referred to simply as rhinitis, was indicated by a response of "Yes, I still have it" to the question, "Have you ever had hay fever or any other allergy that makes your nose runny or stuffy, apart from colds?" The presence of asthma was indicated by an affirmative response to the question, "Have you ever had asthma?" in a subject who claimed to "still have it," admitted to "wheezing or whistling in the chest" that occurred "apart from colds," or had more than rare "attacks of shortness of breath with wheezing."

Allergy skin tests were carried out according to the prick technique,[16] using five tests that included a variety of allergens common to the Tucson area, plus a control. Commercially available skin-test materials were used (supplied by Hollister–Stier Laboratories). The allergens used in the five tests included (1) house dust, 1:10; (2) Bermuda grass, 1:20; (3) tree mix, 1:20 (olive, mulberry, mesquite, and cottonwood); (4) weed mix, 1:20 (rabbit bush, saltbush, careless weed, Russian thistle, desert ragweed, and slender ragweed); and (5) Dematiaceae mold mix, 1:100 (alternaria, helminthosporium, cladosporium, curvularia, spondylocladium, and stemphylium species). After subtraction of the size of the control, each test was graded on a scale (0 to 4) based on the frequency of a reaction of its size in the total population, as previously described.[7] An overall allergy skin-test index was calculated for each subject by summing the individual test scores. The use of this method reduced the possibility that a reaction to any one of the allergens would have an overwhelming influence on the overall test result. A skin-test index of 0 represented completely negative responses to all five tests; the maximum score could reach 20 only if unusually large reactions occurred to all the allergens applied.

Measurement of serum IgE was carried out according to the paper radioimmunosorbent test (PRIST) (Pharmacia Diagnostics, Piscataway, N.J.) method. The reported values represent the mean of duplicate tests and are expressed in international units per milliliter. (It is conventional to express IgE concentrations in this manner, but their value in Système International units — micrograms per liter — can be approximated by multiplying the number of international units by 2.4.[17]) IgE values show a log normal distribution in the general population.[11] For this reason, log IgE was used in all statistical analyses, and average values were reported as geometric means. Serum IgE shows a marked decrease with age and is significantly lower in women than in men. To standardize for age and sex, IgE values were transformed into Z scores, according to sex and the age groups 6 to 14, 15 to 34, 35 to 54, 55 to 74, and 75+ years of age. Age–sex-standardized IgE Z scores indicated the number of standard deviations by which individual subjects differed from the mean for their age–sex-specific group.

To test for the independent effects of IgE level, skin-test reactivity, and smoking habits, as well as their possible interactive effects on the prevalence of asthma and rhinitis, we used two types of logistic models. The first was a qualitative (or categorical) model, in which all relevant independent variables were stratified.[18,19] This technique, in which each stratum is introduced as an independent regression variable, provides maximal flexibility to the multivariate analyses, avoiding a priori assumptions concerning the form of the relation between the independent and dependent variables. Relevant variables were stratified as follows: log IgE — <0.5, 0.5<1, 1<1.5, 1.5<2, 2<2.5, 2.5<3, and 3+; skin-test index — 0, 1 to 3, 4 to 6, 7 to 9, and 10+; and smoking — nonsmokers, current smokers, and exsmokers. Age was stratified as described for the derivation of IgE Z scores. In these analyses, the risk of disease in each stratum was compared with that in the lowest stratum; this base-line stratum was assigned an odds ratio of unity. For skin-test index and smoking, the definitions of these base-line strata were straightforward; they consisted of negative skin test and nonsmoking status, respectively. For serum IgE, the choice of the base-line stratum was dictated by the finding that subjects with log IgE values <0.32 did not have asthma. The predictive value of a given risk factor was assessed by determining the difference in the goodness-of-fit chi-square between models including and excluding the risk factor of interest.[18] The regression coefficient for each level of a particular risk factor was an estimate of the logarithm of the odds ratio that a subject had the disease, as compared with the base-line group.

The second type of logistic analysis that we used was a quantitative linear model.[18] This method was used to test the hypothesis that the log odds ratio of having disease was linearly related to the level of the apparent risk factor. Interactions between different risk factors and between risk factors and potential confounders were also tested. In these analyses, the mean value for the variable in each stratum was attributed to that group after weighting by the number of subjects in each stratum. Data were then standardized, and the Z scores obtained were introduced into the model. This type of analysis yielded a logistic regression coefficient that represents the change in the log odds ratio per unit change in the Z score after adjustment for confounders. Deviation from linearity was tested by determining the difference in goodness-of-fit chi-square between this model and the qualitative one described above. Confidence intervals around the regression of log odds ratios and levels of IgE and the skin-test index were calculated with the use of the weighted analysis proposed by Kleinbaum and Kupper,[20] as modified by Rothman.[19]

Reported probability values are two-tailed. For continuous variables, these values were calculated by analysis of variance, and for discrete variables by chi-square or trend chi-square techniques.

Results

As expected, asthma rates showed highly significant progressive increases with increases in both the skin-test index and serum IgE level ($P<0.0001$). The relation of asthma to IgE was especially close when the latter was expressed as an age–sex-standardized IgE Z score. Regardless of age, none of the 177 subjects with the lowest Z scores (<-1.46) had asthma, whereas 9.6 percent of the remaining 2480 subjects did ($P<0.0001$). The prevalence increased to 37.3 percent among the 67 subjects with IgE Z scores of 2.0 or more.

The overall rates of asthma and rhinitis in three broad age groups are shown in Table 1. The table also shows the frequencies of skin-test reactivity and of

Table 1. Disease Prevalence, IgE Levels, and Skin-Test Index, According to Age Groups.

Subjects	Age 6 to 34	Age 35 to 54	Age 55+
All subjects			
Number	1083	545	1029
Percent with asthma	8.2	8.6	9.8
Percent with rhinitis	42.7	47.3	37.8
Subjects without asthma			
Number	994	498	928
Percent with positive skin test	44.3	40.0	22.8
Percent with IgE >100 IU/ml	30.2	18.1	13.0
Geometric mean IgE (IU/ml)	43	26	18
Mean IgE Z score ±SD*	−0.08 ±0.97	−0.08 ±0.96	−0.07 ±0.98
Subjects with asthma			
Number	89	47	101
Percent with positive skin test	78.7	72.3	39.6
Percent with IgE >100 IU/ml	73.0	57.4	33.7
Geometric mean IgE (IU/ml)	224	117	56
Mean IgE Z score ±SD*	0.90 ±0.87	0.86 ±1.00	0.60 ±1.00

*The differences between the age groups are not significant, but the differences between the subjects with and without asthma in each age group are significant (P<0.0001).

"elevated" IgE concentrations, as well as the geometric mean serum IgE levels and mean IgE Z scores in subjects with and without asthma. The cutoff point for a high IgE level — 100 IU per milliliter — was roughly equivalent to the upper limit of normal recommended in a popular textbook of medicine.[21] These indexes of allergy declined markedly in subjects with asthma who were older than 55, but so did the rates of atopy and the levels of IgE in subjects without asthma.

Although the serum IgE levels in subjects with asthma showed a significant decline with age (P<0.0001), the IgE Z scores showed only a slight and statistically insignificant difference with age. In all age groups, however, both geometric mean IgE levels and IgE Z scores in subjects with asthma were significantly higher than in subjects without asthma (P<0.0001). Figure 1 shows the prevalence of asthma in the three age groups in relation to their age–sex-standardized IgE Z scores. When IgE levels were examined in relation to the overall distribution of IgE values according to age and sex, the relations of the rates of disease to IgE were remarkably similar in all age groups.

A close examination of the overall relations of rhinitis and asthma to the skin-test index and the serum IgE level suggested that the prevalence of asthma seemed to follow the serum level of IgE more closely than the degree of atopy, whereas the opposite appeared to be true of rhinitis. To test the hypothesis that asthma, in contrast to rhinitis, was primarily related to serum IgE and that its relation to the skin-test index might in large part be dependent on the close interrelation between IgE and skin-test reactivity, we performed the logistic analyses described in the Methods section.

After we controlled for age and sex, both the IgE level and the skin-test index were significant predictors of the disease. Both remained significant after adjustment for the effect of the other variable (P<0.0001), but the goodness-of-fit chi-square for IgE (179.9) was higher than that for the skin-test index (53.3). Furthermore, after adjustment for age, sex, and skin-test index, the use of a linear model for the IgE effect did not significantly change the goodness-of-fit of the relation of asthma to IgE levels that was found with the qualitative analysis using seven categories of IgE concentrations. This indicates that asthma rates increase linearly with log IgE values. In this analysis, the logistic regression coefficient (±SE) for the prevalence of asthma in relation to the IgE Z score was 0.76±0.08. This linear relation did not apply, however, to the relation of asthma to the skin-test index. In this case, after adjustment for IgE, the linear model gave significantly worse results than the qualitative model.

A history of smoking did not add to the ability to predict the prevalence of asthma in the logistic analyses when age, sex, serum IgE level, and skin-test index were controlled for, and no significant interactions were noted between smoking and any of the other predictor variables. Also, there were no significant interactions between age or sex and the skin-test index or serum IgE level in determining disease frequencies, or between the skin-test index and serum IgE level.

The relative relations of the skin-test index and IgE level to rhinitis were quite different from their relations to asthma. After we controlled for age and sex, both the skin-test index and the IgE level did remain statistically significant predictors of rhinitis after adjustment for the other (P<0.0001 for skin-test index and P<0.005 for IgE), but the goodness-of-fit chi-square for the skin-test index (158.0) was higher than that for the serum IgE level (20.3). Also, unlike the results of the analysis for asthma, those of the quantitative linear logistic regression model indicated that the relation of the rhinitis rate to the skin-test index was essentially linear; the logistic regression coefficient (±SE) was 0.62±0.05 in this analysis.

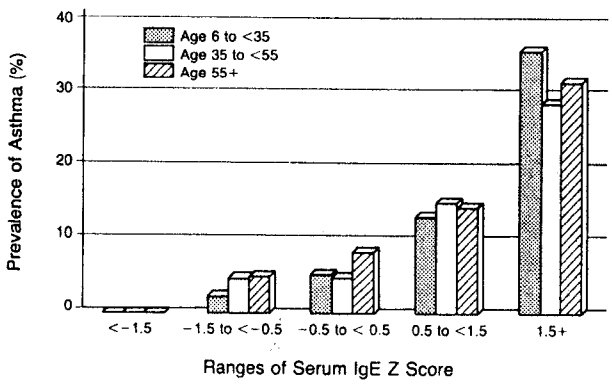


Figure 1. Prevalence of Asthma in Relation to IgE Z Scores Standardized for Age and Sex.

In all age groups, the trend for prevalence to increase with increasing Z scores had a high level of significance (P<0.0001). The following numbers of subjects (according to age group) were at risk in the five ranges of scores: age 6<35 — 73, 248, 435, 252, and 75; age 35<55 — 43, 123, 205, 139, and 35; and age 55+ — 50, 255, 431, 236, and 57.

Figure 2 shows the relation of the odds ratio (on a log scale) of having asthma and rhinitis to the serum level of IgE. For this purpose, we used the regression coefficients obtained from the qualitative logistic analysis of IgE (see Methods) after correction for age, sex, skin-test index, and smoking status. Although there was evidence of colinearity among the regression variables entered into the model and the base-line level for IgE was chosen arbitrarily, the relative position of the increasing levels was still representative of the independent relation between the log odds ratio and risk-factor level. Figure 2 also demonstrates a remarkably linear relation between the log odds ratio of having asthma and increasing levels of IgE, as shown in the statistical analysis. In contrast, the relatively slight independent relation of rhinitis to IgE appeared to be present primarily at higher levels of IgE.

Figure 3 shows an identical type of analysis relating the log odds ratios for both diseases to levels of skin-test reactivity after adjustment for age, sex, serum IgE level, and smoking status. It is obvious that the relative relations of asthma and rhinitis to the skin-test index are reversed from their relative relations to serum IgE. The essentially linear relation of rhinitis to the skin-test index is evident. The independent association of asthma with the skin-test index appears to be related primarily to the presence or absence of a positive test; it shows little progressive increase with higher degrees of skin-test reactivity.

From a clinical viewpoint, it seemed relevant to demonstrate these same relations separately in the subjects who appeared least allergic according to the allergy skin test or the serum IgE level. The relation of asthma rates to the age–sex-standardized IgE Z scores in the 1662 subjects with completely negative

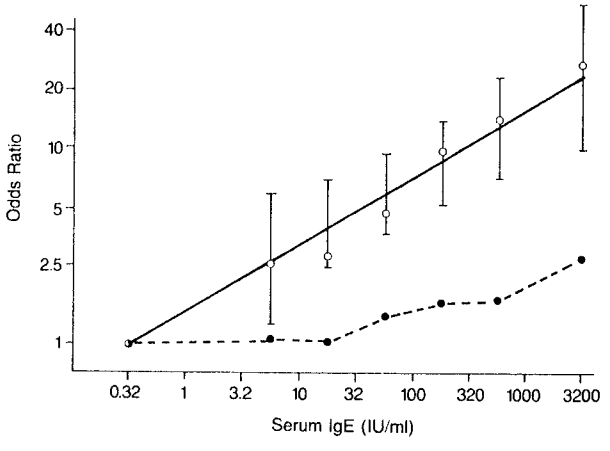


Figure 2. Odds Ratio (Log Scale) of Having Asthma (Open Circles) at Seven Levels of the Total Serum IgE Concentration, after Correction for Age, Sex, Smoking Habits, and Skin-Test Index in a Logistic Analysis.

The solid line, representing the risk of asthma, is a weighted, least-squares linear regression model fitted to the odds ratios at each log IgE level. The vertical lines are 95 percent confidence intervals around the regression for each odds ratio corresponding to a given log IgE level. The dashed line represents the odds ratios of having rhinitis at the same seven levels of serum IgE, after correction for the same confounders.

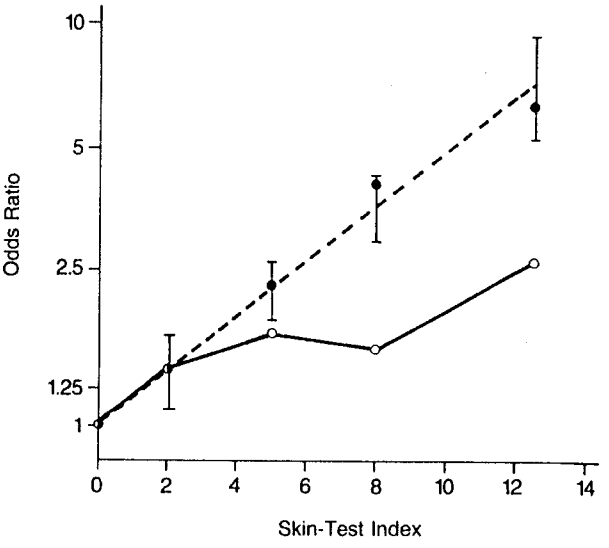


Figure 3. Odds Ratio (Log Scale) of Having Rhinitis (Solid Circles) at Five Levels of the Skin-Test Index after Correction for Age, Sex, Smoking Habits, and Serum IgE in a Logistic Analysis.

The dashed line, representing rhinitis, is a weighted least-squares linear regression model fitted to the odds ratios at each level of the skin-test index. The vertical lines are 95 percent confidence intervals around the regression for each odds ratio corresponding to a given value for the skin-test index. The solid line represents the odds ratio of having asthma at the same five levels of the skin-test index, after correction for the same confounders.

allergy skin tests is shown in Figure 4. If a lack of reactivity to our battery of common aeroallergens is accepted as indicative of an "intrinsic" form of the disease, this graph shows the relation of seemingly intrinsic asthma to the age–sex-standardized IgE level ($P<0.0001$ by trend chi-square). If the findings in an equal number of subjects with the lowest serum IgE levels (the 1662 subjects with IgE Z scores <0.32) are examined, no consistent relation is seen between asthma rates and the degree of allergy skin-test reactivity (Fig. 5), but a significant relation is evident between rhinitis rates and the skin-test index ($P<0.0001$).

Discussion

Our findings in regard to reported "allergic rhinitis" are relatively straightforward. In the Tucson area, 31.2 percent of subjects who have completely negative responses to skin tests of a battery of common aeroallergens report symptoms of rhinitis due to "hay fever or any other allergy," and the rate of reported disease is near 30 percent even in nonatopic subjects who have very low serum IgE levels. We presume that many of these cases represent nonspecific nasal reactions to our dry and somewhat dusty environment. It might be argued that the apparent frequency of atopy (as defined above) in subjects who report that they have rhinitis would be increased if more invasive methods of skin testing were employed. However, it has been shown that reactions to intradermal tests only, and not prick tests, are frequently unassociated with the presence of allergen-specific IgE in the serum.[22,23]

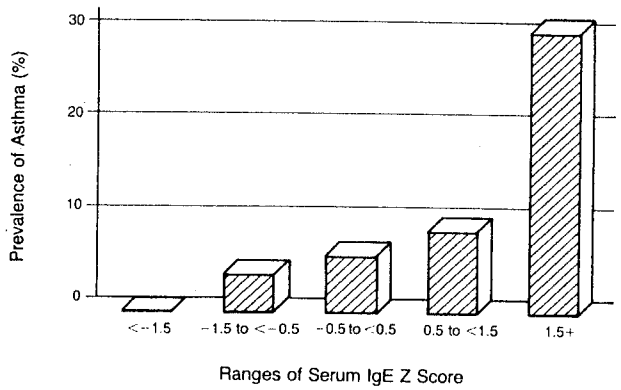


Figure 4. Prevalence of Asthma in Relation to IgE Z Scores, Standardized for Age and Sex, in the 1662 Subjects with Completely Negative Allergy Skin Tests.

The trend for prevalence to increase with increasing Z scores had a high level of significance ($P<0.0001$). The following numbers of subjects were at risk in the five ranges of scores: 150, 521, 689, 259, and 43.

Nonetheless, the rate of reported rhinitis increased markedly with the degree of allergy skin-test reactivity to the antigens applied in this study, exceeding 85 percent among subjects with the most strongly positive tests (skin-test indexes of 16+). The finding that serum IgE levels added relatively little to the predictability of the symptoms of rhinitis after the degree of atopy was taken into account suggests that the battery of skin tests applied adequately represented the local aeroallergens most likely to induce a nasal reaction.

The situation with respect to asthma is much more complex. The clear-cut differences between asthma and rhinitis in their relations to atopy and serum IgE were unexpected, since not only are IgE and atopy closely interrelated but allergic rhinitis and asthma tend to be concordant. Nevertheless, although rhinitis had little relation to serum IgE levels after atopy was accounted for, the opposite was noted in regard to asthma. Asthma rates were closely related to serum IgE levels even in nonatopic subjects, and in all subjects they remained related to IgE after we accounted for the degree of atopy. The consistency of the relations at all ages became clear, however, only after serum IgE levels were considered in relation to the age and sex of the subjects.

Were it not for the findings about rhinitis, one would suspect that the relations to asthma might have reflected only a gross inadequacy of the skin tests used to assess atopy. But if this were the case, the rate of rhinitis should also have been closely related to serum IgE as well as to the skin-test index. It might also be argued that the high IgE Z scores of elderly subjects, which were associated with elevated asthma rates, represented relatively low actual serum levels of IgE and therefore were unlikely to be associated with a significant allergic response. However, the tendency to form IgE is an inherited trait,[24,25] and the serum IgE level has a marked tendency to "track" with age. Even serum IgE levels present at nine months of age are correlated with those found in cord blood at the time of birth.[26] Among the subjects who were entered in the present study before the age of 35, there was a marked and significant decline in the serum IgE level over the ensuing eight years[10]; nevertheless, IgE values recorded during follow-up strongly correlated with those recorded at entry ($r = 0.827$). An almost identical correlation was found in our subjects 35 years old or older at entry ($r = 0.822$), in whom the mean change in IgE level during the eight-year period was insignificant. Thus, high IgE Z scores among elderly subjects were likely to identify those who had much higher levels of IgE earlier in life, when their problems with asthma probably began. In addition, we have found that the presence of as little as 1 IU of Bermuda grass–specific IgE per milliliter of serum is almost always associated with a positive skin-test reaction to this antigen (Halonen M: unpublished data). Large amounts of antigen-specific serum IgE are not necessary for an allergic response.

The most obvious question raised by the present findings is related to the nature of the allergic stimulus that leads to the type of IgE associated with asthma. Comparisons with rhinitis suggest that atopy to the common aeroallergens used in this study is not the essential factor, although one cannot deny that such aeroallergens may induce asthma, and they certainly appear to aggravate the symptoms of existing disease in some patients. Rackemann suggested many years ago that "intrinsic" asthma represented an allergic reaction to bacteria within the respiratory tract.[1] Although pneumococcal-specific IgE can sometimes be demonstrated,[27] there is no firm evidence that allergy to bacteria in the airway is of importance in the pathogenesis of asthma. A number of well-recognized allergens shown to be involved in asthma were not included in our skin-test battery, including dermatophagoides (the house-dust mite) and animal danders. However, dermatophagoides is uncommon in our locale, and when we applied an extract of it to subjects in our population sample on repeat skin test-

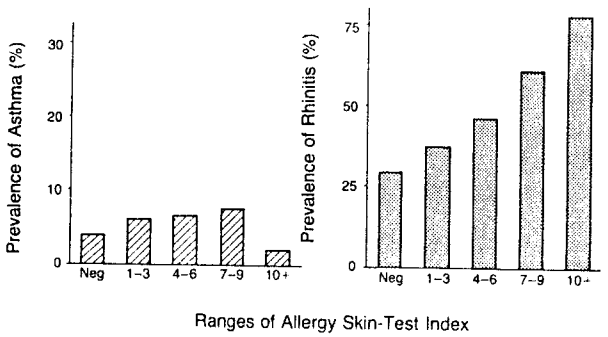


Figure 5. Prevalence of Asthma and Rhinitis in Relation to the Allergy Skin-Test Index in the 1662 Subjects with the Lowest IgE Z Scores (<0.32).

There was no statistically significant trend for asthma rates to increase with the values for the skin-test index in these subjects, but the increase in rhinitis rates had a high level of significance ($P<0.0001$). The following numbers of subjects were at risk in the five ranges for the skin-test index: 1253, 161, 137, 66, and 45.

ing several years after enrollment, it produced even fewer positive reactions than our crude house-dust antigen. In fact, only 2 percent of the retested population reacted to this antigen[28] but not to any of the allergens used in the present study. Also, very low rates of reactions to cat, dog, and horse danders were found in a subset of our population who underwent quite extensive allergy skin testing,[22] especially among subjects who had completely negative reactions to the antigens used in the present study. However, additional organic antigens with possible relevance to asthma are being recognized and reported, such as airborne particles of insect origin.[29] It is also clear from studies of occupational asthma that a wide variety of relatively simple compounds, such as plicatic acid (in workers handling red cedar) and platinum salts,[30,31] are capable of inducing IgE-mediated asthma and that this may occur in otherwise nonatopic subjects. The range of possible allergic stimuli other than commonly recognized aeroallergens is almost limitless. If it were not for the clusters of cases of asthma within occupational groups, many substances now recognized as causes of asthma[32] would never have been identified.

None of the above is meant to imply that a complete evaluation by a trained allergist in a clinical setting might not have detected specific allergens in many of the subjects with asthma who appeared nonatopic on the basis of prick-test reactivity to the aeroallergens used in the present study. On the contrary, the relations to serum IgE suggest that our battery of allergy skin tests was inadequate to detect the wide range of allergens that may be of importance in asthma. We also recognize that our determination of the presence or absence of disease was relatively crude and was based entirely on information from questionnaires. More refined criteria for the diagnosis of asthma and allergic rhinitis in epidemiologic studies would be highly desirable, but any misclassification of cases in the present study would have served only to confound rather than to exaggerate the demonstrated relations to serum IgE and allergy skin-test reactivity.

Regardless of the specific allergic stimulus involved, since no asthma at all was found in subjects with extremely low IgE levels, virtually all the observed cases might be considered to be related to this evidence of "allergy." Indeed, the present data do not support the division of asthma into the extrinsic or allergic type and the intrinsic or nonallergic type. The difference in the course of asthma between older subjects and younger subjects may be the result of many factors wholly unrelated to the allergic origins of their disorder. However, regardless of the level of serum IgE, a minority of subjects have asthma, suggesting that some other factor in host susceptibility may have a critical role in the disease. Inherent hyperresponsiveness of the airways could well represent this essential risk factor,[33] but the present data suggest that some IgE-related reaction is required to induce overt asthma even in susceptible persons. The mechanism for this relation cannot be determined from this type of study. Also, we cannot deny the possibility that there may be unusual forms of asthma that are not associated with IgE, but such cases must be so uncommon that they have not influenced the relations noted in the present report.

References

1. Rackemann FM. A clinical study of one hundred and fifty cases of bronchial asthma. Arch Intern Med 1918; 22:517-52.
2. Ishizaka K, Ishizaka T, Hornbrook MM. Physico-chemical properties of reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. J Immunol 1966; 97:75-85.
3. Johansson SGO, Bennich H. Immunological studies of an atypical (myeloma) immunoglobulin. Immunology 1967; 13:381-94.
4. Rowe DS, Wood CBS. The measurement of serum immunoglobulin E levels in healthy adults and children and in children with allergic asthma. Int Arch Allergy Appl Immunol 1970; 39:1-5.
5. Johansson SGO. Raised levels of a new immunoglobulin class (IgND) in asthma. Lancet 1967; 2:951-3.
6. Ostergaard PA. Non-IgE mediated asthma in children. Acta Paediatr Scand 1985; 74:713-9.
7. Barbee RA, Lebowitz MD, Thompson HC, Burrows B. Immediate skin-test reactivity in a general population sample. Ann Intern Med 1976; 84:129-33.
8. Freidhoff LR, Meyers DA, Bias WB, Chase GA, Hussain R, Marsh DG. A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. I. Epidemiology of reported allergy and skin-test positivity. Am J Med Genet 1981; 9:323-40.
9. Chan-Yeung M, Vedal S, Lam S, Enarson D. Immediate skin reactivity and its relationship to age, sex, smoking, and occupational exposure. Arch Environ Health 1985; 40:53-7.
10. Barbee RA, Halonen M, Kaltenborn W, Lebowitz M, Burrows B. A longitudinal study of serum IgE in a community cohort: correlations with age, sex, smoking, and atopic status. J Allergy Clin Immunol 1987; 79:919-27.
11. Barbee RA, Halonen M, Lebowitz M, Burrows B. Distribution of IgE in a community population sample: correlations with age, sex and allergen skin test reactivity. J Allergy Clin Immunol 1981; 68;106-11.
12. Wittig HJ, Belloit J, De Fillippi I, Royal G. Age-related serum immunoglobulin E levels in healthy subjects and in patients with allergic disease. J Allergy Clin Immunol 1980; 66:305-13.
13. Gerrard JW, Heiner DC, Ko CG, Mink J, Meyers A, Dosman JA. Immunoglobulin levels in smokers and non-smokers. Ann Allergy 1980; 44:261-2.
14. Burrows B, Halonen M, Barbee RA, Lebowitz MD. The relationship of serum immunoglobulin E to cigarette smoking. Am Rev Respir Dis 1981; 124:523-5.
15. Lebowitz MD, Knudson RJ, Burrows B. Tucson epidemiologic study of obstructive lung diseases. I. Methodology and prevalence of disease. Am J Epidemiol 1975; 102:137-52.
16. Pepys J. Skin tests in diagnosis. In: Gell PGH, Coombs RRA, Lachmann PJ. Clinical aspects of immunology. 3rd ed. Oxford: Blackwell Scientific Publications, 1975:55-80.
17. Geha RS. Human IgE. J Allergy Clin Immunol 1984; 74:109-20.
18. Breslow NE, Day NE. Statistical methods in cancer research. Vol. I. The analysis of case-control studies. Lyon, France: International Agency for Research on Cancer, 1980:192-246.
19. Rothman KJ. Modern Epidemiology. Boston: Little, Brown, 1986:327-50.
20. Kleinbaum DG, Kupper LL. Applied regression analysis and other multivariable methods. Boston: Duxbury Press, 1978:243.
21. Braunwald E, Isselbacher KJ, Petersdorf RG, Wilson JD, Martin JB, Fauci AS. Harrison's principles of internal medicine. New York: McGraw-Hill, 1987:A-2.
22. Brown WG, Halonen MJ, Kaltenborn WT, Barbee RA. The relationship of respiratory allergy, skin test reactivity, and serum IgE in a community population sample. J Allergy Clin Immunol 1979; 63:328-35.
23. Reddy PM, Nagaya H, Pascual HC, et al. Reappraisal of intracutaneous tests in the diagnosis of reaginic allergy. J Allergy Clin Immunol 1978; 61:36-41.
24. Bazaral M, Oregel HA, Hamburger RN. IgE levels in normal infants and mothers and an inheritance hypothesis. J Immunol 1971; 107:794-801.
25. Kjellman N-IM, Johansson SGO. IgE and atopic allergy in newborns and infants with a family history of atopic disease. Acta Paediatr Scand 1976; 65:601-7.
26. Halonen M, Dunn AM, Holberg CJ, Wright A, Taussig LM. Total serum IgE in infants: relation to initial cord serum IgE level, age, gender, and lower respiratory infections. Am Rev Respir Dis 1985; 131:A23. abstract.
27. Bloom JW, Halonen M, Dunn AM, Pinnas JL, Burrows B. Pneumococcus-specific immunoglobulin E in cigarette smokers. Clin Allergy 1986; 16:25-32.

28. Barbee RA, Kaltenborn W, Lebowitz MD, Burrows B. Longitudinal changes in allergen skin test reactivity in a community population sample. J Allergy Clin Immunol 1987; 79:16-24.
29. Kino T, Chihara J, Fukuda K, Sasaki Y, Shogaki Y, Oshima S. Allergy to insects in Japan. III. High frequency of IgE antibody responses to insects (moth, butterfly, caddis fly, and chironomid) in patients with bronchial asthma and immunochemical quantitation of the insect-related airborne particles smaller than 10 μm in diameter. J Allergy Clin Immunol 1987; 79:857-66.
30. Tse KS, Chan H, Chan-Yeung M. Specific IgE antibodies in workers with occupational asthma due to western red cedar. Clin Allergy 1982; 12:249-58.
31. Cromwell O, Pepys J, Parish WE, Hughes EG. Specific IgE antibodies to platinum salts in sensitized workers. Clin Allergy 1979; 9:109-17.
32. Chan-Yeung M, Lam S. Occupational asthma. Am Rev Respir Dis 1986; 133:686-703.
33. Boushey HA, Holtzman MJ, Sheller JR, Nadel JA. Bronchial hyperreactivity. Am Rev Respir Dis 1980; 121:389-413.

ABNORMALITIES IN PARATHYROID HORMONE SECRETION AND 1,25-DIHYDROXYVITAMIN D_3 FORMATION IN WOMEN WITH OSTEOPOROSIS

SHONNI J. SILVERBERG, M.D., ELIZABETH SHANE, M.D., LUZ DE LA CRUZ, R.N., GINO V. SEGRE, M.D., THOMAS L. CLEMENS, PH.D., AND JOHN P. BILEZIKIAN, M.D.

Abstract We investigated the parathyroid hormone–1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D) axis in osteoporosis by administering phosphate to 8 postmenopausal women with osteoporosis (49 to 78 years old) and to 10 normal women matched for age (50 to 74 years). All subjects responded with a similar increase in the serum phosphorus concentration (women with osteoporosis, 1.15±0.06 to 1.79±0.09 mmol per liter; controls, 1.14±0.05 to 1.73±0.08 mmol per liter) and a fall in the ionized calcium concentration (women with osteoporosis, 1.12±0.03 to 1.06±0.03 mmol per liter; controls, 1.17±0.01 to 1.11±0.02 mmol per liter).

Parathyroid hormone levels rose 2.5-fold in the control group (15.4±2.2 to 37.9±6.1 pg per milliliter) but increased by only 43 percent in the group with osteoporosis (14.8±2.8 to 21.2±4.1 pg per milliliter), an increase similar to that previously reported in young normal subjects (53 percent). In healthy older and younger subjects, the levels of 1,25(OH)$_2$D did not change; in the subjects with osteoporosis, however, they decreased significantly (50 percent).

We conclude that older women require a greater parathyroid hormone stimulus than younger women to maintain vitamin D homeostasis, because of an age-related decline in the formation of 1,25(OH)$_2$D in response to parathyroid hormone, and that in osteoporosis the age-appropriate parathyroid hormone response to the same hypocalcemic signal is diminished. Our results are consistent with the presence of an abnormality in parathyroid hormone secretory function in osteoporosis in addition to the universal decline in 1,25(OH)$_2$D responsiveness associated with aging. (N Engl J Med 1989; 320:277-81.)

IT is now well established that parathyroid hormone is a key regulator of the renal production of 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D).[1-3] Parathyroid hormone stimulates the renal l-α-hydroxylase enzyme, leading to increased conversion of 25-hydroxyvitamin D to the active form of the hormone, 1,25(OH)$_2$D. Responsiveness to this regulatory pathway appears to become impaired in osteoporosis and aging, resulting in reduced formation of the active vitamin D metabolite.[4-7] A possible mechanism of altered 1,25(OH)$_2$D formation in osteoporosis is a primary defect in the renal l-α-hydroxylase or, alternatively, a defect in factors that stimulate the enzyme. These potential abnormalities in 1,25(OH)$_2$D formation in osteoporosis, however, have not been distinguished from the alteration in 1,25(OH)$_2$D production that is known to be a feature of the process of aging.

To address these issues, we studied the parathyroid hormone–1,25(OH)$_2$D axis in women with osteoporosis, using a challenge with oral phosphate. There has been considerable interest recently in the use of phosphate as part of sequential-treatment protocols in the therapy of osteoporosis.[8,9] For the purpose of this study, however, the use of phosphate allowed us to assess the effect of opposing stimuli to 1,25(OH)$_2$D production: the direct effect of phosphate in suppressing 1,25(OH)$_2$D levels, and an indirect effect of phosphate through elevation of parathyroid hormone levels in stimulating the formation of 1,25(OH)$_2$D.[10,11] With the use of age-matched control women without osteoporosis, the protocol permitted an examination of both endogenous parathyroid hormone responsiveness and 1,25(OH)$_2$D production in women with osteoporosis. Data on a group of young normal subjects who were studied according to an identical protocol[12] enabled us also to evaluate aging as a factor independent of osteoporosis. The results support the existence of a distinct abnormality in the parathyroid hormone–1,25(OH)$_2$D axis in osteoporosis.

From the Departments of Medicine (S.J.S., L.C., E.S., J.P.B.), Pharmacology (J.P.B.), and Pathology (T.L.C.), College of Physicians and Surgeons, Columbia University, New York; Regional Bone Center, Helen Hayes Hospital, West Haverstraw, New York (T.L.C.); and the Endocrine Unit, Massachusetts General Hospital, Boston (G.V.S.). Address reprint requests to Dr. Silverberg at the Department of Medicine 9-410, College of Physicians & Surgeons, 630 W. 168th St., New York, NY 10032.

Supported in part by grants (DK-01836, AR-39191, DK-32333, and RR-00645) from the National Institutes of Health. Dr. Silverberg is the recipient of a Clinical Investigator Award from the National Institutes of Diabetes, Digestive and Kidney Diseases.

Presented in part at the Tenth Annual Meeting of the American Society of Bone and Mineral Research, New Orleans, June 1988.

METHODS

We studied 8 women with osteoporosis (mean age, 63 years; range, 49 to 78) and 10 age-matched controls (mean age, 61; range 50 to 74). All subjects were postmenopausal white women. Those with osteoporosis had at least two nontraumatic vertebral compression fractures, and their bone mineral density as measured by dual-photon absorptiometry of the lumbar spine was more than 2 SD below the mean expected for their age. In five of

ALLERGY Net

ALLERGY 2005: 60: 702–710

Bacillus clausii effects in children with allergic rhinitis

G. Ciprandi*, A. Vizzaccaro, I. Cirillo, M. A. Tosca

Key words: allergic rhinitis; antihistamines; *Bacillus calusii*; children; eosinophils; probiotics.

Allergic rhinitis is characterized by T-helper (Th)2 polarization as elevated levels of Th2-derived cytokines, including interleukin (IL)-4, IL-5 and IL-13, have been evidenced at nasal level in allergic rhinitics (1). Th2 cytokines play a pathogenic role as they induce IgE synthesis and eosinophil infiltration. Th2 polarization in allergic subjects may occur as consequence of reduced pressure of microbial agents in the gut: the so-called Hygiene Hypothesis (2). Probiotics may stimulate immune system at all mucosal surfaces and exert a primary prevention of atopic diseases and reduce allergic symptoms and inflammatory parameters (3). We previously found out that *Bacillus clausii* was capable of modulating cytokine pattern at nasal level in allergic children with recurrent respiratory infections (4). Particularly, *B. clausii* restored physiological Th1 polarization and induced T-regulatory cell response, as documented by increased levels of IL-10 and tumor growth factor (TGF)β after treatment (4).

***Bacillus clausii* modulates nasal allergic inflammation cooperating with antiallergic drugs.**

Therefore, we conducted a pilot study to investigate the potential effects exerted by *B. clausii* on nasal symptoms, eosinophils, and symptomatic use of antihistamines in children with allergic rhinitis.

Twenty allergic children, 13 males and seven females, with an average age of 13.4 years (range 12–15) were consecutively evaluated. A detailed clinical history and a complete physical examination, including allergy evaluation, were carried out for each patient. The diagnosis of allergic rhinitis due to pollen sensitization was made according to validated criteria (5). The study was blinded both to the investigator who performed the nasal eosinophil assessment and the investigator who carried out statistical analysis.

Symptomatic use of levocetirizine (5 mg tablets) was prescribed for all children. Ten of them were randomly treated with oral *B. clausii* (Enteroger-

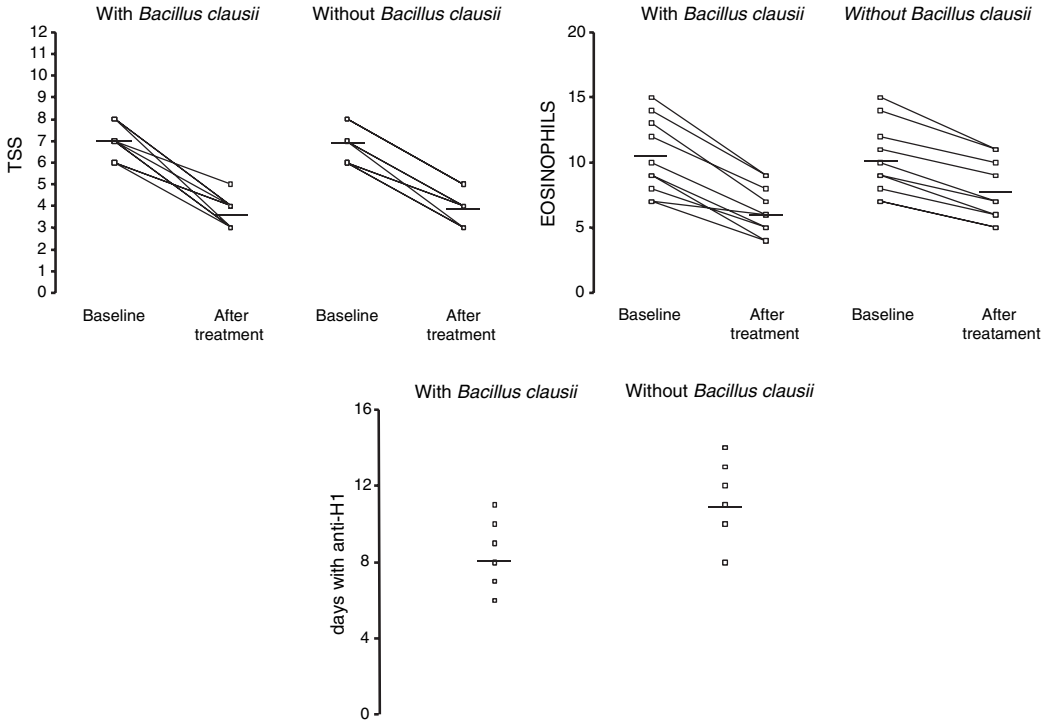


Figure 1. TSS in children before and after treatment with or without *Bacillus clausii* (left upper panel). Eosinophil number in children before and after treatment with or without *B. clausii* (right upper panel). Number of days with antihistamine assumption (lower panel).

mina, Sanofi-Synthelabo OTC, Milan, Italy) spores (2 billion spores/vial) at the dosage schedule of three vials a day for 3 weeks.

The study was performed during the pollen season, when patients were symptomatic, and was approved by the Institutional Review Board. Informed consent was obtained from the parents of the children. Nasal total symptoms score (TSS), nasal eosinophils, and number of days associated with antihistamine assumption were evaluated. Symptoms and eosinophils were assessed in all subjects at baseline and after 3 weeks according to validated criteria (6).

The data collected were subjected to statistical analysis by MANOVA and *post hoc* comparison by Duncan's test. All results with P-values < 0.05 obtained were considered statistically significant.

Children treated with *B. clausii* spores, showed significant reduction ($P = 0.049$) of TSS without any side-effect: 7 ± 0.8 at baseline, and 3.7 ± 0.7 after treatment. Nasal eosinophils significantly ($P = 0.048$) diminished: 10.4 ± 2.9 at baseline, and 6.3 ± 1.9 after treatment.

Children treated only with levocetirizine on demand did not show significant ($P = 0.051$) reduction of TSS: 6.9 ± 0.9 at baseline, and 4 ± 0.8 after 3 weeks. Moreover, nasal eosinophils were not significantly ($P = 0.69$) diminished: 10.2 ± 2.8 at baseline, and 7.7 ± 2.3 after 3 weeks.

The intergroup analysis showed that symptom relief was not significantly different in two groups ($P = 0.061$), whereas eosinophils were significantly less in children treated with probiotic ($P = 0.042$). Finally, children treated with *B. clausii* spores significantly ($P = 0.034$) assumed less levocetirizine tablets than other subjects: 8.1 ± 1.7 *vs* 11.1 ± 2.2 days (Fig. 1).

In conclusion, this pilot study provides evidence that *B. clausii* may exert a modulatory effect on allergic response as documented by reduced eosinophil infiltration. Moreover, this probiotic may synergize with antihistamine in relieving nasal symptoms. In addition, the symptomatic use of antihistamine does not allow adequate control of both symptoms and inflammation. The possible mechanism of action of *B. clausii* may be attributed to the previous documented activity on immune response at the nasal level (4).

*Allergologia-U.O. ORL
Padiglione Specialità (piano terzo)
Ospedale San Martino
Largo R. Benzi 10
16132 Genoa
Italy
Tel: 00 39 10 5552124
Fax: 00 39 10 5556682
E-mail: gio.cip@libero.it

Accepted for publication 27 July 2004
Allergy 2005: 60:702–703

DOI: 10.1111/j.1398-9995.2005.00722.x

References

1. Christodoupoulos P, Cameron L, Durham S, Hamid Q. Molecular pathology of allergic disease. II: Upper airway disease. J Allergy Clin Immunol 2000;**105**:211–223.
2. Bjorksten B. The intrauterine and postnatal environments. J Allergy Clin Immunol 1999;**104**:1119–27.
3. Matricardi P, Bjorksten B, Bonini S, Bousquet J, Djukanovic R, Dreborg S et al. Microbial products in allergy prevention and therapy. Allergy 2003;**58**:461–471.
4. Ciprandi G, Tosca MA, Ricca V, Passalacqua G, Riccio AM, Bagnasco M et al. *Bacillus clausii* exerts immunomodulatory activity in allergic children with recurrent respiratory infections: a pilot study. Ped Allergy Immunol 2004;**15**:148–451.
5. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma (ARIA). Allergy 2002;**57**:841–855.
6. Ciprandi G, Tosca MA, Milanese M, Caligo G, Ricca V. *Bacillus clausii* exerts immuno-modulatory activity in allergic children with recurrent respiratory infections: a pilot study. Ped Allergy Immunol 2004;**15**:148–151.

Flow-assisted diagnosis of anaphylaxis to patent blue

D. G. Ebo, R. D. Wets, T. K. Spiessens, C. H. Bridts, W. J. Stevens*

Key words: anaphylaxis; CD63; flow cytometry; methylene blue; patent blue.

A 20-year-old man was referred because of an anaphylactic reaction with angioedema, bronchospasm with cyanosis and profound hypotension (systolic pressure 120 mmHg to immeasurable) during anaesthesia for combined excision of a dermal cyst in the neck and a multifistulated sacral cyst. History revealed no previous allergies. Revision of his anaesthetic report disclosed that the reaction had started 30 min after induction with propofol, sufentanyl and cisatracurium, but within minutes after fistulography with patent blue (Blue patente V®, Guerbet, Aulnay, France).

We report a case of patent blue-induced anaphylaxis during fistulography in which flow cytometry confirmed diagnosis.

Laboratory analysis showed a normal blood count, complement profile and protease inhibitor concentrations. Total immunoglobulin E (IgE) was 11 kU/l and IgE for latex, suxamethonium and ethylene oxide were negative (< 0.35 kUa/l; Immuno-CAP FEIA method; Pharmacia Diagnostics, Brussels, Belgium). Skin tests included latex (Stallergenes, Genval, Belgium), chlorhexidine-digluconate, serial dilutions of five muscle relaxants: suxamethonium (Myoplegine®), pancuronium (Pavulon®), rocuronium (Esmeron®), atracurium (Tracrium®) and cisatracurium (Nimbex®), the analgesic sufentanyl (Sufenta®), the anaesthetic propofol (Diprivan®), and the dyes patent and methylene blue. Except for patent blue (Table 1) skin tests were negative.

AMERICAN JOURNAL OF INDUSTRIAL MEDICINE 46:404–407 (2004)

Brief Report

IgE Sensitization to Bacterial and Fungal Biopesticides in a Cohort of Danish Greenhouse Workers: The BIOGART Study

Gert Doekes, PhD,[1]* Preben Larsen, MD,[2] Torben Sigsgaard, MD,[3] and Jesper Baelum, MD[2]

Background *The use of biopesticides in agriculture may implicate new risks of work-related allergic reactions.*
Methods *Sera were tested from the BIOGART project, a longitudinal respiratory health study among >300 Danish greenhouse workers. IgE was measured by enzyme immunoassay (EIA) with extracts of biopesticide products containing Bacillus thuringiensis (BT) or Verticillium lecanii (Vert).*
Results *Many sera had detectable IgE to BT (23–29%) or Vert (9–21%). IgE titers from the 2- and 3-year follow-up (n = 230) were highly correlated, with discordant results in <15%. IgE titers to different BT, or to different Verticillium products were also significantly correlated (both r > 0.70), whereas IgE anti-BT and anti-Verticillium showed no correlation at all.*
Conclusions *Exposure to these microbial biopesticides may confer a risk of IgE-mediated sensitization. In future research there is a need to identify allergenic components in the preparations, perform studies on non-exposed controls and analyze the relation between sensitization and health parameters.* Am. J. Ind. Med. 46:404–407, 2004.

KEY WORDS: IgE; biopesticides; greenhouse workers; allergy

INTRODUCTION

The use of biologic pest control in agriculture has shown a rapid increase in the last few decades. Its beneficial effects, as an alternative for chemical pesticides, are obvious: not only would it markedly reduce the adverse ecological effects of environmental pollution with chemical pesticides and the risks of pesticide residue exposure in the general public, it also would strongly diminish the hazards of occupational exposure to toxic pesticides and fungicides among agricultural workers. The most intensive use of biologic pest control takes place in greenhouses and horticulture, where there is a high susceptibility to plant diseases and insect pests due to the high crop density and specific climate conditions.

One of the (many) strategies in biologic pest control involves the use of insect pathogens: bacterial or fungal micro-organisms that more or less specifically infect and kill certain taxonomic groups of harmful insects like white flies or lice, while being harmless to most other invertebrates. A typical example is *Bacillus thuringiensis* (BT): a bacterium producing a potent enterotoxin that is mainly released in the gut of insects that have swallowed the BT bacteria [Schnepf et al., 1998]. An analogous fungal insect pathogen is *Verticillium lecanii* (Vert), which is mainly used to prevent and combat crop damage caused by aphids [Askary et al., 1999].

[1]Institute for Risk Assessment Sciences, University Utrecht, The Netherlands
[2]Department of Occupational and Environmental Medicine, Odense University Hospital, Denmark
[3]Department of Environmental and Occupational Medicine, University of Aarhus, Denmark 3508
Presented at the 4th Skokloster Workshop Conference: Organic Dusts—Agents, Disease and Prevention, Gothenborg, Sweden, April 7–10, 2003.
Contract grant sponsor: Danish Environmental Protection Agency.
*Correspondence to: Dr. Gert Doekes, Institute for Risk Assessment Sciences, University Utrecht, PO Box 80176, 3508 TD Utrecht, The Netherlands. E-mail: g.doekes@iras.uu.nl

Accepted 26 July 2004
DOI 10.1002/ajim.20086. Published online in Wiley InterScience (www.interscience.wiley.com)

The working mechanism of these agents, however, requires active dispersion of relatively large amounts of living or viable bacterial or fungal spores. The commercially available bacterial or fungal biopesticides are usually supplied as dried powders with a high microbial cell content, or as concentrated suspensions. Before application, suspensions are prepared or diluted in water in simple open buckets or other vessels from which the diluted suspensions are dispersed via a tubing system and aerosolized in the greenhouse. While the actual dispersal can be done in the absence of any workers in the greenhouse, exposure may occur after re-entry as dermal exposure when touching plants or other surfaces, and inhalation either of microbial cells that may remain airborne for prolonged periods, or of cells made airborne by the workers' activities.

So far, neither exposure levels nor the potentially associated health effects of biopesticide use have been systematically investigated. Possible adverse health effects could be either 'non-specific' airway inflammation, or specific type I, III, or IV allergic inflammation caused by allergen-specific sensitization. One study in the US has reported type I sensitization in a relatively small group of BT-exposed agricultural workers, as measured by skin prick tests and IgE serology [Bernstein et al., 1999], while no effects have as yet been reported for *Verticillium* use. In this study, the prevalence of specific IgE sensitization to extracts of commercially available preparations of BT and *Verticillium* was assessed among Danish greenhouse workers.

MATERIALS AND METHODS

Population

The serologic study was performed as part of the BIOGART study [Larsen et al., 2002]: a longitudinal epidemiologic study in a cohort of >300 Danish greenhouse workers, with a specific focus on respiratory health, including the prevalence and incidence of common and work-related respiratory symptoms, lung function status and changes, and development of bronchial hyper-responsiveness. As part of the study, venous blood samples have been taken at baseline and after 1, 2, and 3 years of follow-up, to measure total IgE and IgE to specific allergens, and to assess IgE sensitization to potential work-related allergens, particularly bio-pesticide components. This report contains the results for the 2nd year of follow-up, for which data was available, from 216 women (mean age 36.6 ± 10.2 yr) and 113 men (aged 36.3 ± 11.3 yr).

IgE Serology

Sera were tested for the presence of IgE to extracts of 8–10 different bio-pesticide preparations, including two products containing spores of *Verticillium* (Mycotal® and Vertalec®) and two BT-based products (Bactimos® and Vectobac®). These biopesticides will be further designated as Vert-M, Vert-V, BT-B, and BT-V, respectively. Other biopesticides included in the study were products containing spores of *Trichoderma harzianum* (Supresivit®) or *Paecilomyces spp.* (Preferal®) and other BT preparations. According to the responses to yearly questionnaires on exposure, these products were much less used in the participating greenhouse companies, and accordingly, sensitization was rare. We, therefore, here only report the IgE anti-Vert and anti-BT reactions.

Extracts from dry powders (Vert-M and Vert-V) were made essentially as described earlier for the preparation of, e.g. wheat or potato antigen/allergen preparations [Houba et al., 1996; Zock et al., 1996]: 5% (w/v) suspensions were incubated overnight at room temperature in phosphate-buffered saline (PBS) containing 0.02% sodium azide, and the next day ultrasonicated for 15 min, followed by vigorous shaking for 30 min. Biopesticides supplied as suspension (BT-B and BT-V) were first centrifuged (30 min at 2,750*g*), and the supernatants were harvested and left overnight at room temperature. From the pellets, 3 g (wet weight) was mixed with 30 ml PBS-azide, and extracted overnight as described above for the 5% (w/w) powder suspensions, including ultrasonication and vigorous shaking for 30 min.

Insoluble material was removed by centrifugation (15 min; 2,100*g*) and filtration through paper filter and Millipore 0.45 μm filters. The final extracts were extensively dialyzed against PBS-azide and stored in aliquots at -20°C. Protein concentrations in the extracts were determined with the BCA method (Pierce, Rockford, IL).

Serum IgE antibodies to biopesticides were assessed by a previously described enzyme immunoassay (EIA) method [Doekes et al., 1996]. Microtiter plates were coated overnight by incubation with the extracts in PBS-azide at 20 μg protein per milliliter. After blocking with PBS containing 0.05% Tween-20 and 0.2% gelatin (PBTG), sera diluted 1/10 in PBTG were added and incubated for 2 hr at 37°C. After thorough washing of the plates with PBS-Tween, bound IgE was detected with a four-step procedure including mouse monoclonal anti-human IgE, biotinylated affinity-purified rabbit anti-mouse Ig, avidin-peroxidase, and finally *o*-phenylenediamine (OPD) plus H_2O_2. The peroxidase reaction was stopped with 1 N HCl and the optical density read at 492 nm.

All sera were tested at a 1/10 dilution in duplicate wells for each test allergen/biopesticide extract, and in two non-coated wells to account for non-specific IgE binding to the plates, while in each plate also for each extract two wells were included with PBTG instead of serum ('no serum control'). The average OD_{492} values in allergen-coated wells were corrected both for the OD_{492} values in the non-coated wells, and for the OD_{492} of the no-serum controls. Sera were considered positive if the corrected OD_{492} was >0.05 [Doekes et al., 1996].

TABLE I. Positive Serum IgE Reactions to Extracts of Bio-Pesticides in the 2nd Year Follow-Up of the BIOGART Study (n = 329)

Biopesticide	Preparation	No. positive sera	Percent
Verticillium lecanii	Vert-M (Mycotal®)	28	8.5
	Vert-V (Vertalec®)	58	17.6
	Vert-M and Vert-V	23	7.0
	Vert-M and/or Vert-V	63	19.1
Bacillus thuringiensis	BT-B (Bactimos®)	78	23.7
	BT-V (Vectobac®)	72	21.9
	BT-B and BT-V	60	18.2
	BT-B and/or BT-V	90	27.4

RESULTS

Table I gives a summary of the IgE EIA reactions for the four biopesticide preparations. High prevalences of sensitization were found for both *Verticillium*- and BT-based products: from 8.5% for *Verticillium*-M to 23.5% for BT-B. Positive reactions for the two *Verticillium* products were closely associated, with only 5 of the 23 IgE anti-Vert-M positive sera being negative on Vert-V, and 13 of the 35 IgE anti-Vert-V positive sera being negative on Vert-M (OR 35.0 and 95% CI 12.5–97.9). IgE anti-BT reactions showed an even more pronounced association, with more than 80% of sera reacting to either BT-B or BT-V also being positive on the other preparation (OR = 68.4 and 95% CI 30.3–145.3). In contrast, sensitization to *Verticillium* products on the one hand, and to BT on the other hand, showed no or only weak, non-significant associations, with ORs ranging from 1.4 to 1.8 (data not shown).

IgE reactions to extracts from products containing the same micro-organism were also quantitatively associated, as shown by the strong correlations between OD_{492} values of 'double-positive' sera (Fig. 1a,b). These graphs further illustrate that the large majority of all positive reactions were relatively weak: only a few sera showed IgE anti-Vert-V and/or Vert-M reactions with OD_{492} values around 1.0 or higher, and IgE anti-BT positive sera showed, with one exception, only OD_{492} values <0.2.

From approximately two-thirds of the population a serum sample from the 3rd follow-up year was also available, and comparison of 2nd and 3rd year IgE serologic results could be made for 229–236 subjects for whom valid test results were available for both years. As shown by Table II, prevalences of positive IgE reactions in year 3 were very similar to those in year 2 (Table I), and a very close association was found between 2nd and 3rd year EIA results, with concordance levels of >90% and high positive and negative predictive values. Also highly significant quantitative correlations were found when the OD_{492}** of 2nd and 3rd year IgE EIA tests for individuals positive in both years, were plotted and compared (with Pearson's $r > 0.8$ for log-transformed OD_{492} values).

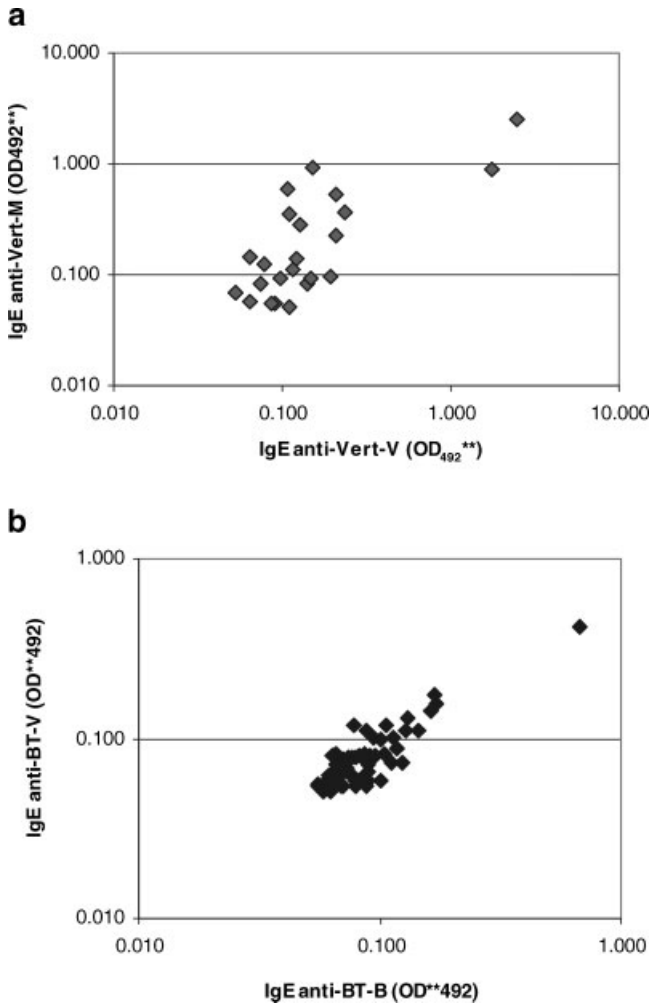


FIGURE 1. Correlation between OD_{492} values (**) of positive IgE reactions to extracts of (**a**) two *Verticillium lecanii* (Vert) preparations: Vert-M (Mycotal®) and Vert-V (Vertalec®) (**b**) two *Bacillus thuringiensis* (BT) preparations: BT-B (Bactimos®) and BT-V (Vectobac®) (**) OD_{492} values for 1/10 diluted sera, after correction for non-specific IgE binding to EIA plates, and for background reactions in 'no serum' control wells.

DISCUSSION

The results suggest that regular use of microbial biopesticides may be associated with a risk of biopesticide-specific IgE sensitization. There has previously been concern regarding the potentially negative health effects of exposure to BT, but most studies in this area have focused on the risk of infectious disease. In one study the prevalence of positive skin prick tests and of IgE reactions to BT extracts was investigated in a population of approximately 100 agricultural workers with estimated high or low levels of BT exposure [Bernstein et al., 1999]. While no evidence was found for work-related respiratory, possibly allergic symptoms, a significantly higher prevalence of positive SPTs and IgE tests was found among highly exposed farm workers. Present data are in line with these findings. In addition,

TABLE II. IgE Anti-Biopesticide Reactions: Comparison of 2nd and 3rd Year Follow-Up (N = 229–236)

Biopesticide	Positive sera in 3rd year (%)	Concordance between 2nd and 3rd year results (%)	PPV[a] (%)	NPV[a] (%)
Vert-M	22/230 (9.5)	96	80	97
Vert-V	49/232 (21.1)	90	77	93
BT-B	62/236 (26.3)	86	80	88
BT-V	47/229 (20.5)	88	73	91

[a]PPV and NPV: Positive and negative predictive value of the 2nd year IgE test results.

another biopesticide, derived from *Verticillium* was identified as a possibly equally potent sensitizer.

Since the whole crude high molecular weight fractions extracted from the biopesticide products were used, the IgE reactions in the EIAs may be primarily directed against non-active carrier proteins, contaminants, etc. On the other hand, the strong correlations between IgE reactions against the two different BT products, and between the IgE reactions against two different *Verticillium* products, strongly suggest that in both cases the IgE antibodies are mainly directed against a genuine BT and genuine *Verticillium* allergenic HMW component. Nevertheless, the actual specificities of the IgE immune responses have to be studied in more detail, as well as their relation with exposure levels. At least, more extensive data series should be collected on IgE sensitization in non-exposed controls. Even while the IgE binding components may be genuine BT and *Verticillium* components, they could also be shared with some or many commonly found bacterial or fungal species, and in that case sensitization in the general population may be expected as well.

Another important issue may be the development of sensitization during repeated and/or continuous exposure at the workplace. We previously tested large numbers of sera from the first two years (years 0 and 1) of the cohort study, and found lower prevalences of IgE sensitization, while in the present series IgE EIA results for sera taken at year 2 and 3 of the follow-up showed a very close correlation (Table II). For three of the four products the prevalence of sensitization was slightly higher in year 3, which might mean that (a) while most sensitization takes place after 1–2 years of work-related exposure, some workers may be sensitized only after prolonged exposure; and (b) that an established specific IgE response may develop in subsequent years, especially when exposure continues.

It should be emphasized, however, that these reported results are of preliminary nature and that further analyses are required before definitive conclusions regarding the risks of occupational allergy can be drawn. While there was a relatively high prevalence of various airway and other symptoms in the studied cohort [Larsen et al., 2002], a first crude analyses did—just as in the earlier US study on BT sensitization [Bernstein et al., 1999]—not show a clear-cut direct association with a type I sensitization. A further analysis of the biopesticide extracts, and particularly identification and characterization of the actual IgE binding components is an important and necessary next step to confirm the allergenicity of these agents.

In summary, these IgE serologic data may be a reason for concern that frequent use of microbial pesticides is a risk factor for occupational IgE-mediated allergic sensitization. Further studies focusing on both the specificity of these IgE reactions, and the association with work-related symptoms, are required to come to more definite conclusions.

ACKNOWLEDGMENTS

We thank the greenhouse companies and the workers for their collaboration and willingness to participate in the study. We appreciate the skillful technical assistance of Mrs. Griet Terpstra and Mr. Jack Spithoven who prepared the allergen extracts and performed the IgE EIAs, and of Lutzen Portengen, MSc, who helped during elaboration and evaluation of EIA data.

REFERENCES

Askary H, Benhaou N, Brodeur J. 1999. Ultrastructural and cytochemical characterization of aphid invasion by the hyphomycete *Verticillium lecanii*. J Invertebr Pathol 74:1–13.

Bernstein IL, Bernstein JA, Miller M, Tierzieva S, Bernstein DI, Lummus Z, Selgrade MK, Doerfler DL, Seligy VL. 1999. Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. Environ Health Perspect 107:575–582.

Doekes G, Douwes J, Wouters I, de Wind S, Houba R, Hollander A. 1996. Enzyme immunoassays for total and allergen specific IgE in population studies. Occup Environ Med 53:63–70.

Houba R, Van Run P, Heederik D, Doekes G. 1996. Wheat antigen exposure assessment for epidemiological studies in bakeries using personal dust sampling and inhibition ELISA. Clin Exp Allergy 26:154–163.

Larsen P, Sigsgaard T, Doekes G, Baelum J. 2002. Results from a 1-year follow-up study of the health effects of three types of microbiological pesticides. The 12th Eur Respiratory Society, Annual Congress). Eur Resp J 20(suppl 38):121s (abstract).

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806.

Zock JP, Doekes G, Heederik D, Van Zuylen M, Wielaard P. 1996. Airborne dust antigen exposure and specific IgG response in the potato processing industry. Clin Exp Allergy 26:542–548.

© International Epidemiological Association 2002 Printed in Great Britain International Journal of Epidemiology 2002;31:1098–1102

REVIEW

Asthma and the westernization 'package'

Jeroen Douwes[a,b] and Neil Pearce[a]

Ten years ago we knew what caused asthma, and we knew how to prevent it. Asthma was an atopic disease caused by allergen exposure. The fundamental aetiological mechanism was that allergen exposure, particularly in infancy, produced atopic sensitization and continued exposure resulted in asthma through the development of eosinophilic airways inflammation, bronchial hyper-responsiveness and reversible airflow obstruction. Asthma prevalence was increasing around the world because of changes in lifestyle and domestic building design that were increasing allergen exposure. The solution was therefore clear: to prevent asthma we needed to prevent exposure to allergens.[1–3]

In recent years it has become increasingly evident that this picture is, at best, too simplistic.[4] Bronchial responsiveness is a poor surrogate measure of clinical asthma and the current evidence is that it has lower validity than standard symptom questionnaires.[5] Less than one half of asthma cases are attributable to atopy and/or eosinophilic airways inflammation,[6] and (non-allergic/non-atopic) neutrophilic airways inflammation may account for the other half.[7] Furthermore, although there are some clear cases of allergen exposure causing asthma in adults in the occupational environment, overall there is little evidence that allergen exposure is a major primary cause of asthma,[8] and even some evidence that allergen exposure early in life may have a protective effect (see below).

The 'established' risk factors for asthma, including allergen exposure, were 'discovered' primarily on the basis of clinical studies and case reports of exacerbations in asthma patients. It is natural for physicians and patients to assume that the factors involved in secondary causation may also be important for primary causation. Once the theory became established it was easy to find 'verifications' of the allergen hypothesis, and to dismiss 'refutations' as chance findings or as being due to inadequate measurements of exposure and outcome. Of course, there are well-documented instances where allergen exposure does act as a primary cause of asthma, particularly in adults. However, allergen exposure does not appear to be the major primary cause of asthma that it has been assumed to be, nor to account for global patterns of asthma prevalence, or the striking increases in asthma prevalence over time.[9]

[a] Centre for Public Health Research, Massey University Wellington Campus, Private Box 756, Wellington, New Zealand.

[b] Institute for Risk Assessment Sciences (IRAS), Division of Environmental and Occupational Health, Utrecht University, The Netherlands.

Correspondence: Jeroen Douwes, Centre for Public Health Research, Massey University Wellington Campus, Private Box 756, Wellington, New Zealand. E-mail: j.douwes@massey.ac.nz

Westernization and asthma

As the 'established' asthma risk factors are increasingly being called into question, epidemiological studies are playing a major role in the search for new theoretical paradigms which are more consistent with the epidemiological evidence and which have greater explanatory power. Recent research has therefore shifted attention from allergens that may cause sensitization and/or provoke asthma attacks, to factors that may 'programme' the initial susceptibility to asthma, through allergic or non-allergic mechanisms. This also in part involves a shift of attention from risk factors for asthma to protective factors, and the possible role of the loss of protective factors in the global increases in asthma prevalence.

Several studies have indicated that certain exposures (in particular of microbial origin) early in life may protect against atopy and asthma which has led to the 'hygiene hypothesis'. Briefly, microbial exposure may affect T lymphocytes which have an important function in controlling immune responses including (amongst others) help for B cell production of antibodies (IgE, IgG, IgA, IgM). T-helper 2 (TH_2) cells stimulate B-cells to produce IgE upon allergen stimulation whereas T helper 1 cells (TH_1) inhibit this process. In these processes various cytokines are involved including TH_1-associated interleukin 12 (IL-12) and interferon gamma (IFN-γ), and IL-5 and IL-4 associated with TH_2 immunity (Figure 1). The so called 'hygiene hypothesis' postulates that growing up in a more hygienic environment with less microbial exposure may enhance atopic (TH_2)

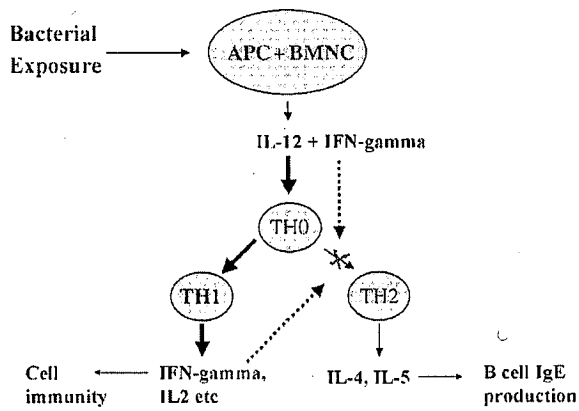


Figure 1 Potential protective effects of microbial exposure by enhancing TH1 and inhibiting TH2 immunity

APC: Antigen presenting cells; BMNC: Blood mononuclear cells; IL: interleukin; IFN-gamma; interferon-gamma; TH: T helper cell; IgE; Immunoglobulin E.

immune responses, whereas microbial pressure would drive the response of the immune system—which is known to be skewed in an atopic TH_2 direction during fetal and perinatal life—into a TH_1 direction and away from its tendency to develop atopic immune responses.[10,11]

This 'hygiene hypothesis' has been prompted by the results of several epidemiological studies showing that overcrowding and unhygienic conditions were associated with a lower prevalence of atopy, eczema and hay-fever although the evidence for asthma is less consistent.[12,13] A large number of siblings (especially older siblings) and attendance at day care centres were determined to be particularly protective.[14,15] An increase in infections has been proposed as an explanation for these findings.[16]

Several studies have shown a direct association between infections (e.g. hepatitis A, measles) or immunization with BCG and a lower prevalence of atopy and allergies.[17–20] However, the results for airborne viruses (measles, mumps, rubella and chickenpox) and BCG vaccination were inconsistent.[21–24] The role of respiratory viral infections is also not very clear; respiratory tract infections are known to exacerbate pre-existing asthma,[25] and lower respiratory tract infections may be a risk factor for asthma.[26,27] On the other hand upper respiratory infections may protect against atopy and asthma.[27] The type of infection (food-borne and oral-faecal versus airborne, and viral versus bacterial) and location (higher versus lower airways) may thus be important in determining the effect on atopy and asthma. Furthermore, exposure to specific microbial agents with strong pro-inflammatory properties such as bacterial endotoxin have been suggested to be protective.[11,28–30] One study demonstrated in infants that endotoxin levels in house dust correlated with IFN-γ producing T cells (TH_1), but not with IL-4, IL-5 or IL-13 producing cell proportions (TH_2). In addition, it was shown that allergen sensitized infants had significantly lower endotoxin levels in house dust than non-sensitized infants.[31] However, the evidence for a protective effect of endotoxin is currently still weak.[32]

In addition to specific agents with potential protective effects, subpopulations have been identified with low atopy and asthma rates compared to the general populations. For instance, it has been documented that children with an anthroposophic lifestyle in Sweden[33] and children raised on farms with livestock in Europe, Canada, and Australia[34–41] have less atopy and asthma. However, it is currently not clear which specific factors confer protection but specific microbial exposures have been hypothesized to be involved either through ingestion (lactobacilli) or inhalation (endotoxin; see above). Ingestion of lactobacilli may be important since they have the ability to colonize the human gut[42] and some (mostly experimental) evidence exists suggesting that they may modify the immune development into a non-atopic TH_1 direction.[43–50] However, various other microbes of the gut flora may play a role as well.[51–53]

Finally, several studies have shown that the presence of pets in the home early in life in Europe was negatively associated with atopy.[54,55] This should, however, be interpreted with caution, since avoidance behaviour (removal of pets in the families with sensitized and/or symptomatic children) may have contributed to this inverse association.[56] Recently, an explanation was offered for a potential protective effect by Platts-Mills *et al.*[57,58] who suggested some form of tolerance involving a modified TH_2 response characterized by the presence of high IgG4 antibody levels and suppressed IgE production. Alternatively, increased exposures to bacterial endotoxin may play a role since it is known that pets in the home are associated with higher endotoxin levels in house dust.[59–62] In other parts of the world (Guinea-Bissau and Nepal) it has been shown that pigs and cattle in the home are associated with less atopy.[20,63] This is in line with observations that animal contact in farmers' children may confer protection, and as hypothesized for the farmers' children, increased endotoxin exposures may play a role.[32]

Thus the current 'hygiene hypothesis', may explain an increase in atopy and *allergic* asthma. However, with the large proportion of asthma that is not associated with atopy it is questionable whether the 'hygiene hypothesis' (as defined above) on its own can explain the large increase observed over the last decades. For instance, in a repeated population survey among preschool children an increase in asthma prevalence was not only found in children with the classic asthma pattern of wheeze but in all wheezing phenotypes including viral wheeze.[64] However, this was not confirmed in another study among children and young adults that showed an increase in all wheezers but not in viral wheeze. In addition, some studies have suggested that only atopic asthma has increased, but in those studies poor markers of atopy were used with unknown validity.[65,66] Studies in farmers' children have indicated that protective effects of farming were independent from effects on atopic sensitization.[36] Finally, although housing conditions have likely not become more hygienic in US inner city populations, asthma prevalence has increased significantly in those populations, and particularly among African Americans living in poverty,[67,68] which is in contrast to previous findings showing a positive association between affluence and asthma prevalence. These studies thus further emphasize the potential limitations of the current hygiene hypothesis. However, whatever mechanism is involved, it is becoming increasingly clear that the 'package' of changes associated with westernization may be contributing to the global increases in asthma prevalence, and that this process involves an increase in asthma susceptibility rather than an increase in exposure to 'established' asthma risk factors.[9]

Fetal growth and asthma

In this context, it is of interest that some recent studies have identified a relatively large size at birth as a risk factor for the subsequent development of asthma in adolescence,[69–72] and the findings of Yuan *et al.*[73] in this issue of the *International Journal of Epidemiology* add to this evidence. These studies have examined the 'other end' of the 'Barker hypothesis' which had previously almost exclusively focussed on the 'low end' of the fetal growth spectrum which was found to be associated with an increased risk of disease in adult life. Now we are discovering that bigger is not always better, and that there are also health consequences of high fetal growth. The reasons for these associations are unclear, but fetal growth apparently reflects a number of intrauterine influences, particularly relating to maternal diet and placental function. This is potentially a finding of major importance since anthropometric measures at birth have increased during this century.

Nevertheless, it is important to place these findings in perspective. Firstly, the findings are, as usual, inconsistent. A large prospective birth cohort in Finland of 5192 subjects who were

followed up to the age of 31 showed an inverse association between atopy and gestational age (excluding those infants that were born prematurely), but no association was found with length and weight at birth. In addition, no associations with any of the birth characteristics (including birthweight, length and gestational age) and asthma was found.[74] The most consistent finding is that a large head circumference at birth is associated with an increased risk of atopy,[69,71,72] but the association with asthma is much weaker[71,72] with the exception of one study.[70] On the other hand, birth length has been associated with asthma, but not with atopy in one study,[71] while low birthweight has been associated with a reduced risk of asthma in some studies[70,71] and an increased risk in others.[75,76]

Secondly, assuming that the findings reported by Yuan *et al.*,[73] regarding high birthweight and/or ponderal index and subsequent hospitalization for asthma, are confirmed in further studies, they may not be due to the atopic aetiological mechanisms that they focus on in their discussion. As noted above, it appears that at most one half of asthma cases are attributable to atopy,[6] which may explain in part the inconsistent findings for fetal growth, atopy, and asthma.

Finally, it should be noted that the population attributable risk of high fetal growth as a cause of asthma is relatively small. The strongest finding reported by Yuan *et al.*[73] is for birthweight and hospitalization for asthma, but even in this instance the population attributable risk is around 1.5%. As countries become more 'Western', babies are getting larger, and this is associated with an increased risk of atopy and/or asthma, but it cannot explain the striking increases in asthma prevalence over recent decades.

Currently little is known about other prenatal risk factors for atopy and asthma (with the possible exception of maternal smoking), and even less is known about potential protective factors, whereas prenatal events or exposures may be very important in the development of these conditions.[77,78] The cytokine profile of the mother may affect the development of the fetal immune system.[79,80] Prenatal allergen exposure may also play a role.[81] It has been hypothesized, and some evidence has been presented,[82–88] that the immune system may be primed through transamniotic or transplacental exposure to antigens or antigen-derived peptides, resulting in a decreased capacity of TH_1 cytokine interferon γ production, which may result in atopic TH_2 immune responses in neonates.[87] A recent study showed a positive association between allergen exposure (measured as allergen levels in the mother's mattresses) and total IgE in cord blood suggesting that prenatal exposures may indeed affect the development of fetal immune responses.[88] Moreover, in the same study an inverse association was shown for endotoxin. Interestingly, in a recent cross-sectional study among farmers' children it was suggested that farm activity of the pregnant mother (associated with high endotoxin exposures) had a protective effect on atopy and asthma of her child.[36] Thus, prenatal endotoxin exposure could confer protection for atopy and asthma. However, as with pre-natal allergen exposure as a potential risk factor, the evidence is currently weak and needs confirmation in larger prospective studies.

The westernization 'package'

The striking increases in asthma prevalence globally cannot be primarily due to genetic factors, since they are occurring too rapidly, and therefore they must be occurring due to changes in environmental exposures. It seems that as a result of this 'package' of changes in the intrauterine and infant environment, we are seeing an increased susceptibility to the development of asthma and/or allergy. There are a number of elements of this 'package' including changes in maternal diet, increased fetal growth, smaller family size, reduced infant infections and increased use of antibiotics and immunization, all of which have been (inconsistently) associated with an increased risk of childhood asthma, but none of which can alone explain the increases in prevalence.[9] Thus, it is important that we consider the 'forest' of changes that occur with westernization, as well as doing studies of specific 'trees'. In particular, the inconsistent findings with regard to fetal growth and asthma suggest that studies of this issue alone are unlikely to yield major benefits, although they may be an important part of the process of understanding the bigger picture. It is likely that the 'package' is more than the sum of its parts, and that these social and environmental changes are all pushing infants' immune systems in the same direction. To know what that direction is, and which components of the 'package' are responsible, requires that better aetiological theories of asthma are developed to replace the allergen theory, or to incorporate it as a special case.

Although have we focussed in this editorial on perinatal factors and early life exposures that may play a role in programming asthma susceptibility, later exposures (Western or otherwise) may be important too. However, currently little is known (with the exception of occupational asthma) on how exposures later in life may affect these processes. Similarly, most of the studies we have discussed relate to allergic mechanisms for asthma, because few studies have been done on non-allergic mechanisms for asthma and their potential contribution to population trends.

It is essential that in the future as much attention is paid to non-allergic (non-eosinophilic) mechanisms for asthma as has been paid to allergic (eosinophilic) mechanisms in the past. Furthermore, it requires that epidemiologists rigorously test these new theories systematically in population-based studies, rather than the *ad hoc* and anecdotal approach that has been adopted in the past with respect to studies of the allergen hypothesis. Epidemiology has played a major role in calling the 'established' theory of asthma causation into question. It also has a major role to play in developing and testing new aetiological theories of asthma causation.

Acknowledgements

The Centre for Public Health Research is supported by a Programme Grant from the Health Research Council of New Zealand. Jeroen Douwes is supported by a research fellowship from the Netherlands Organization for Scientific Research (NWO).

References

[1] Custovic A, Smith A, Woodcock A. Indoor allergens are a primary cause of asthma. *Eur Respir J* 1998;**53**:155–58.

[2] Peat JK, Tovey E, Toelle BG *et al.* House dust mite allergens: a major risk factor for childhood asthma in Australia. *Am J Respir Crit Care Med* 1996;**153**:141–46.

[3] Platts-Mills TAE, Sporik RB, Chapman MD, Heymann PW. The role of domestic allergens. In: *The Rising Trends in Asthma*. Ciba Foundation Symposium 206. Chichester: Wiley, 1997, pp.173–89.

[4] O'Donnell RA, Frew AJ. Is there more than one inflammatory phenotype in asthma? *Thorax* 2002;**57**:566–67.

[5] Pekkanen J, Pearce N. Defining asthma in epidemiological studies. *Eur Respir J* 1999;**14**:951–57.

[6] Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax* 1999;**54**:268–72.

[7] Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002;**57**:643–48.

[8] Pearce N, Douwes J, Beasley R. Is allergen exposure the major primary cause of asthma? *Thorax* 2000;**55**:424–31.

[9] Pearce N, Douwes J, Beasley R. Asthma. In: Detels R, McEwen J, Beaglehole R, Tanaka H (eds). *Oxford Textbook of Public Health. 4th Edn.* Oxford: Oxford University Press, 2002, vol. 3, pp. 1255–77.

[10] Holt PG, Sly PD, Björkstén B. Atopic versus infectious diseases in childhood: a question of balance? *Pediatr Allergy Immunol* 1997;**8**: 53–58.

[11] Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. *Lancet* 1999;**354**:12–15.

[12] Strachan DP. Hay fever, hygiene and household size. *BMJ* 1989; **299**:1259–60.

[13] Strachan DP. Allergy and family size: a riddle worth solving. *Clin Exper Allergy* 1997;**27**:235–36.

[14] Krämer U, Heinrich J, Wjst M, Wichmann HE. Age of entry to day nursery and allergy in later childhood. *Lancet* 1999;**353**:450–54.

[15] Ball TN, Castro-Rodriguez JA, Griffith KA *et al.* Siblings, day care attendance, and the risk of asthma and wheezing during childhood. *N Engl J Med* 2000;**343**:538–43.

[16] Martinez FD. Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? *Thorax* 1994;**49**: 1189–91.

[17] Matricardi PM, Rosmini F, Ferrigno L *et al.* Cross sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. *BMJ* 1997;**314**:999–1003.

[18] Shaheen SO, Aaby P, Hall AJ *et al.* Cell mediated immunity after measles in Guinea-Bissau: historical cohort study. *BMJ* 1996;**313**: 969–74.

[19] Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997; **275**:77–79.

[20] Aaby P, Shaheen SO, Heyes CB *et al.* Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin Exp Allergy* 2000;**30**:644–50.

[21] Matricardi PM, Rosmini F, Riondino S *et al.* Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* 2000;**320**:412–17.

[22] Alm JS, Lilja G, Pershagen G, Scheynius A. Early BCG vaccination and development of atopy. *Lancet* 1997;**350**:400–03.

[23] Alm JS, Lilja G, Pershagen G, Scheynius A. BCG vaccination does not seem to prevent atopy in children with atopic heredity. *Allergy* 1998; **53**:537.

[24] Gruber C, Kulig M, Bergmann R *et al.* Delayed hypersensitivity to tuberculin, total immunoglobulin E, specific sensitization, and atopic manifestation in longitudinally followed early Bacille Calmette-Guerin-vaccinated and nonvaccinated children. *Pediatrics* 2001;**107**:E36.

[25] Johnston SL, Pattemore PK, Sanderson G *et al.* Community study of role of virus infections in exacerbations of asthma in 9–11 year old children. *BMJ* 1995;**310**:1225–29.

[26] Stein RT, Sherill D, Morgan WJ *et al.* Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 1999;**354**:541–45.

[27] Illi S, von Mutius E, Lau S *et al.* Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. *BMJ* 2001;**322**:390–95.

[28] Von Mutius E, Braun-Fahrlander C, Schierl R *et al.* Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000;**30**:1230–34.

[29] Liu AH, Leung YM. Modulating the early allergic response with endotoxin. *Clin Exp Allergy* 2000;**30**:1535–39.

[30] Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 2000;**22**:604–12.

[31] Gereda JE, Leung DYM, Thatayatikom A *et al.* Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 2000; **355**:1680–83.

[32] Douwes J, Pearce N, Heederik D. Does environmental endotoxin exposure prevent asthma? *Thorax* 2002;**57**:86–90.

[33] Alm JS, Swartz J, Lilja G, Scheynius A, Pershagen G. Atopy in children of families with an anthroposophic lifestyle. *Lancet* 1999;**353**: 1485–88.

[34] Braun-Fahrländer CH, Gassner M, Grize L *et al.* Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. *Clin Exp Allergy* 1999; **29**:28–34.

[35] Riedler J, Eder W, Oberfeld G *et al.* Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* 2000;**30**:194–200.

[36] Riedler J, Braun-Fahrlander C, Eder W, Schreuer M. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;**358**:1129–33.

[37] Von Ehrenstein OS, Von Mutius E, Illi S *et al.* Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000;**30**: 187–93.

[38] Ernst P, Cormier Y. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Resp Crit Care Med* 2000;**161**: 1563–66.

[39] Downs SH, Marks GB, Mitakakis TZ, Koskenvuo M. Having lived on a farm and protection against allergic diseases in Australia. *Clin Exp Allergy* 2001;**31**:570–75.

[40] Kilpeläinen M, Terho O, Helenius H *et al.* Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* 2000; **30**:201–08.

[41] Portengen L, Sigsgaard T, Omland O *et al.* Low prevalence of atopy in young Danish farmers and farming students born and raised on a farm. *Clin Exp Allergy* 2002;**32**:247–53.

[42] Johansson ML, Molin G, Jeppsson B *et al.* Administration of different Lactobacillus strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. *Appl Environ Microbiol* 1993;**59**:15–20.

[43] Sepp E, Julge K, Vasar M *et al.* Intestinal microflora of Estonian and Swedish infants. *Acta Paediatr* 1997;**86**:956–61.

[44] Shida K, Makino K, Morishita A *et al. Lactobacillus casei* inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *Int Arch Allergy Immunol* 1998;**115**: 278–87.

[45] Shida K, Takahashi R, Iwadate E *et al. Lactobacillus casei* strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin Exp Allergy* 2002;**32**:563–70.

[46] Murosaki S, Yamamoto Y, Ito K *et al.* Heat-killed *Lactobacillus plantarum* L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. *J Allergy Clin Immunol* 1998;**102**:57–64.

[47] Björkstén B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999;**29**:342–46.

[48] Kalliomaki M, Slaminen S, Arvilommi H *et al.* Probiotics in primary prevention of atopic diseases: a randomised placebo-controlled trial. *Lancet* 2001;**357**:1076–79.

[49] Hessle C, Hanson LA, Wold AE. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. *Clin Exp Immunol* 1999;**116**:276–82.

[50] Pessi T, Sutas Y, Hurme M, Isolauri E. Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. *Clin Exp Allergy* 2000;**30**:1804–08.

[51] Bottcher MF, Nordin EK, Sandin A, Midtvedt T, Björkstén B. Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clin Exp Allergy* 2000;**30**:1590–96.

[52] Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar T. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;**108**:516–20.

[53] Kalliomaki M, Kirjavainen P, Eerola E *et al.* Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001;**107**:129–34.

[54] Svanes C, Jarvis D, Chinn S, Burney P. Childhood environment and adult atopy: Results from the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 1999;**103**:415–20.

[55] Hesselmar N, Aberg N, Aberg B, Eriksson-B, Björkstén B. Does early exposure to cat or dog protect against allergy development? *Clin Exp Allergy* 1999;**29**:611–17.

[56] Anyo G, Brunekreef B, de Meer G *et al.* Early, current and past pet ownership: associations with sensitization, bronchial responsiveness and allergic symptoms in school children. *Clin Exp Allergy* 2002;**32**: 361–66.

[57] Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet* 2001;**357**:752–56.

[58] Platts-Mills TA, Vaughan JW, Blumenthal K, Pollart Squillace S, Sporik RB. Serum IgG and IgG4 antibodies to Fel d 1 among children exposed to 20 microg Fel d 1 at home: relevance of a nonallergic modified Th2 response. *Int Arch Allergy Immunol* 2001;**124**:126–29.

[59] Douwes J, Zuidhof A, Dockes G *et al.* β(1→3)-glucan and endotoxin in house dust and peak flow variability in children. *Am J Resp Crit Care Med* 2000;**162**:1348–54.

[60] Heinrich J, Gehring U, Douwes J *et al.* Pets and vermin are associated with high endotoxin levels in house dust. *Clin Exp Allergy* 2001;**31**: 1839–45.

[61] Park JH, Spiegelman DL, Gold DR, Burge HA, Milton DK. Predictors of airborne endotoxin in the home. *Environ Health Perspect* 2001;**109**: 859–64.

[62] Gereda JE, Klinnert MD, Price MR, Leung DY, Liu AH. Metropolitan home living conditions associated with indoor endotoxin levels. *J Allergy Clin Immunol* 2001;**107**:790–96.

[63] Melsom T, Brinch L, Hessen JO *et al.* Asthma and indoor environment in Nepal. *Thorax* 2001;**56**:477–81.

[64] Kuehni CE, Davis A, Brooke AM, Silverman M. Are all wheezing disorders in very young (preschool) children increasing in prevalence. *Lancet* 2001;**357**:1821–25.

[65] Russell G, Helms PJ. Trend in occurrence of asthma among children and young adults. Reporting of common respiratory and atopic symptoms has increased. *BMJ* 1997;**315**:1014–15.

[66] Upton MN, McConnachie A, McSharry C *et al.* Intergenerational 20 year trends in the prevalence of asthma and hay fever in adults: the Midspan family study surveys of parents and offspring. *BMJ* 2000;**321**:88–92.

[67] Weiss KB, Gergen PJ, Crain EF. Inner-city asthma: the epidemiology of an emerging US public health concern. *Chest* 1992;**101(Suppl.6)**: 362S–67S.

[68] Crater DD, Heise S, Perzanowski M *et al.* Asthma hospitalization trends in Charleston, South Carolina, 1956 to 1997: twenty-fold increase among black children during a 30-year period. *Pediatrics* 2001;**108**:E97.

[69] Godfrey KM, Barker DJP, Osmond C. Disproportionate fetal growth and raised IgE concentration in adult life. *Clin Exp Allergy* 1994;**24**:641–48.

[70] Fergusson DM, Crane J, Beasley R, Horwood LJ. Perinatal factors and atopic disease in childhood. *Clin Exp Allergy* 1997;**27**:1394–401.

[71] Leadbitter P, Pearce N, Cheng S *et al.* Relationship between fetal growth and the development of asthma and atopy in childhood. *Thorax* 1999; **54**:905–10.

[72] Gregory A, Doull I, Pearce N *et al.* The relationship between anthropometric measurements at birth: asthma and atopy in childhood. *Clin Exp Allergy* 1999;**29**:330–33.

[73] Yuan W, Basso O, Sorensen HT, Olsen J. Fetal growth and hospitalization with asthma during early childhood: a follow-up study in Denmark. *Int J Epidemiol*: 2002;**31**:1240–45.

[74] Pekkanen J, Xu B, Järvelin M-R. Gestational age and occurrence of atopy at age 31—a prospective birth cohort study in Finland. *Clin Exp Allergy* 2001;**31**:95–102.

[75] Shaheen SO, Sterne JAC, Montgomery SM, Azima H. Birth weight, body mass index and asthma in young adults. *Thorax* 1999;**54**: 396–402.

[76] Svanes C, Omenaas E. Birth characteristics and asthma symptoms in young adults: results from a population-based cohort study in Norway. *Eur Respir J* 1998;**12**:1366–70.

[77] Björkstén B. Allergy priming early in life. *Lancet* 1999;**353**:167–68.

[78] Jones CA, Holloway JA, Warner JO. Does atopic disease start in foetal life? *Allergy* 2000;**55**:2–10.

[79] Warner JA, Jones CA, Williams TJ, Warner JO. Maternal programming in asthma and allergy. *Clin Exp Allergy* 1998;**28**:35–38.

[80] Hertz U, Joachim R, Ahrens B *et al.* Prenatal sensitization in a mouse model. *Am J Respir Crit Care Med* 2000;**162**:S62–65.

[81] Warner JA, Jones AC, Miles EA, Warner JO. Prenatal sensitisation. *Pediatr Allergy Immunol* 1996;**7**:98–101.

[82] Prescott SL, Macaubas C, Holt BJ *et al.* Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998;**160**:4730–37.

[83] Szepfalusi Z, Lobihler C, Pichler J *et al.* Direct evidence for transplacental allergen transfer. *Pediatr Res* 2000;**48**:404–07.

[84] Szepfalusi Z, Pichler J, Elsädder S *et al.* Transplacental priming of the human immune system with environmental allergens can occur early in gestation. *J Allergy Clin Immunol* 2000;**106**:530–36.

[85] Holloway JA, Warner JO, Vance GH *et al.* Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. *Lancet* 2000;**356**:1900–02.

[86] Miller RL, Chew GL, Bell CA *et al.* Prenatal exposure, maternal sensitisation, and sensitisation *in utero* to indoor allergens in an inner-city cohort. *Am J Respir Crit Care Med* 2001;**164**:995–1001.

[87] Prescott SL, Holt PG, Jenmalm M, Bjorksten B. Effects of maternal allergen-specific IgG in cord blood on early postnatal development of allergen-specific T-cell immunity. *Allergy* 2000;**55**:470–75.

[88] Heinrich J, Bolte G, Höscher B *et al.* Allergen and endotoxin on mothers' mattresses and total IgE in cord blood of neonates. *Eur Resp J*; in press.

Malaria Journal

BioMed Central

Research

Open Access

Total and functional parasite specific IgE responses in *Plasmodium falciparum*-infected patients exhibiting different clinical status

Joana Duarte[1], Prakash Deshpande[2], Vincent Guiyedi[3,4], Salah Mécheri[5], Constantin Fesel[1], Pierre-André Cazenave[3], Gyan C Mishra[2], Maryvonne Kombila[4] and Sylviane Pied*[1,3]

Address: [1]Instituto Gulbenkian de Ciencia, LEA CNRS-IGC, Oeiras, Portugal, [2]National Centre for Cell Sciences, Pune, India, [3]Unité d'Immunophysiopathologie Infectieuse, Institut Pasteur, 25 rue du Docteur Roux, 75 724, Paris Cedex 15, France, [4]Département de Parasitologie-Mycologie-Médecine Tropicale, Faculté de Médecine de Libreville, Gabon and [5]Unité des Réponses Immunes Précoces aux Parasites, Institut Pasteur Paris, France

Email: Joana Duarte - jduarte@igc.gulbenkian.pt; Prakash Deshpande - pdeshpande@nccs.in; Vincent Guiyedi - guidivin@pasteur.fr; Salah Mécheri - mecheri@pasteur.fr; Constantin Fesel - cfesel@igc.gulbenkian.pt; Pierre-André Cazenave - cazenave@pasteur.fr; Gyan C Mishra - gcishra@nccs.in; Maryvonne Kombila - valentine_favry@yahoo.fr; Sylviane Pied* - spied@pasteur.fr

* Corresponding author

Published: 04 January 2007

Malaria Journal 2007, **6**:1 doi:10.1186/1475-2875-6-1

Received: 25 October 2006
Accepted: 04 January 2007

This article is available from: http://www.malariajournal.com/content/6/1/1

Abstract

Background: There is an increase of serum levels of IgE during *Plasmodium falciparum* infections in individuals living in endemic areas. These IgEs either protect against malaria or increase malaria pathogenesis. To get an insight into the exact role played by IgE in the outcome of *P. falciparum* infection, total IgE levels and functional anti-parasite IgE response were studied in children and adults, from two different endemic areas Gabon and India, exhibiting either uncomplicated malaria, severe non cerebral malaria or cerebral malaria, in comparison with control individuals.

Methodology and results: Blood samples were collected from controls and *P. falciparum*-infected patients before treatment on the day of hospitalization (day 0) in India and, in addition, on days 7 and 30 after treatment in Gabon. Total IgE levels were determined by ELISA and functional *P. falciparum*-specific IgE were estimated using a mast cell line RBL-2H3 transfected with a human Fcε RI α-chain that triggers degranulation upon human IgE cross-linking. Mann Whitney and Kruskall Wallis tests were used to compare groups and the Spearman test was used for correlations.

Total IgE levels were confirmed to increase upon infection and differ with level of transmission and age but were not directly related to the disease phenotype. All studied groups exhibited functional parasite-specific IgEs able to induce mast cell degranulation *in vitro* in the presence of *P. falciparum* antigens. Plasma IgE levels correlated with those of IL-10 in uncomplicated malaria patients from Gabon. In Indian patients, plasma IFN-γ , TNF and IL-10 levels were significantly correlated with IgE concentrations in all groups.

Conclusion: Circulating levels of total IgE do not appear to correlate with protection or pathology, or with anti-inflammatory cytokine pattern bias during malaria. On the contrary, the *P. falciparum*-specific IgE response seems to contribute to the control of parasites, since functional activity was higher in asymptomatic and uncomplicated malaria patients than in severe or cerebral malaria groups.

Background

Malaria is a complex disease that kills between one and two million people every year. Most of those affected are children under five years of age, non-immune individuals and pregnant women [1]. The principal cause of death is infection by *Plasmodium falciparum* due to its ability to induce severe complications such as severe anaemia and/or cerebral malaria (CM) often associated with hypoglycaemia [2-4]. The physiopathology of malaria cannot be represented by a single scheme. For example, patients who develop CM present a range of acute neurological manifestations and the disease is characterized by a diffuse encephalopathy, altered levels of consciousness, deep coma and seizure leading to death. Even though during the last few years a lot of information has become available from clinical and experimental studies, the causes of CM remain to be determined. The clinical outcome of a *P. falciparum* infection depends on the genetic factors of the host and parasite, and also on host immune responses. Antibodies and T cells are among the immune factors thought to play a role in mediating protection and also pathology [2-5].

P. falciparum infection increases the serum levels of IgM and IgG antibodies but also IgE in individuals living in endemic areas [6-12]. IgEs may protect against or participate in malaria pathogenesis. The association of high anti-*P. falciparum* IgE levels with a reduced risk of developing clinical malaria suggests the involvement of IgE in protection [13,14]. The observation that circulating levels of IgE most often correlate with severe rather than uncomplicated disease suggests a pathogenic role of IgE [8,10-12], and the positive correlation between the levels of IgE/IgE immune complexes and the levels of TNF in CM patients provides supporting evidence [8,10-12]. The exact role played by IgE in malaria is still unclear.

IgE is an immunoglobulin isotype that only exists in mammals. It is present at very low concentrations in the serum of normal individuals, at levels ranging from 10 to 300 ng/ml [9]. Its functional effect has been shown to depends on Fc receptors expressed on mast cells and basophils both in mice and humans, as well as on eosinophils, monocytes/macrophages and platelets in humans [9]. IgEs positively regulate both of their receptors: the high affinity receptor (Fcε RI) and the low affinity receptor (Fcε RII or CD23) [15]. The Fcε RI is expressed only on mast cells and/or basophils in both mice and humans [9,16]. The binding of IgE to the high affinity receptor on the mast cell membrane and its subsequent aggregation with antigens results in degranulation and the release of mediators that further aggravate an ongoing allergic process [17]. On basophils, the cross-linking of Fcε RI-bound IgE rapidly induces the release of IL-4 and IL-13 [16], among other inflammatory mediators. The low affinity receptor (Fcε RII) is the second major and widely distributed IgE receptor. It is also known as CD23 and is constitutively expressed on B cells and is induced by IL-4 on macrophages, some T cells, human eosinophils and platelets [9,16]. The cross-linking of CD23 on macrophages or on other CD23-bearing effector cells by IgE-containing immune complexes is thought to play a pathogenic role in malaria via TNF-mediated pathways [16].

This study aimed to evaluate the total and functional *P. falciparum*-specific IgE responses, the association of these responses with plasma cytokine patterns and the phenotype of the disease in endemic controls and infected patients with different clinical forms of malaria. The infected patients originated from a low endemic area in India and a high endemic area in Gabon.

Materials and methods

Study population

Patients from Gabon

All the patients included in this study were children aged between 0.1 and 6 years (mean age = 2.6 years) recruited between 1996 and 2000 at the Owendo Pediatric Hospital (OPH) and the Libreville Hospital Center (LHC) in Gabon (see Table 1). Informed parental consent had been obtained. Gabon has an equatorial climate that is hot and humid, with an endemic malaria transmission. The study design was approved by the local health office ethics committee. The patients were distributed into different groups according to World Health Organization (WHO) guidelines for the definition of uncomplicated and severe malaria [18]. A cohort of 135 *P. falciparum*-infected children was constituted and divided into three groups according to disease severity [[6], 67] 50patients with uncomplicated malaria (UM), 29 with severe non-cerebral malaria (SM) developing severe anaemia (haemoglobin level < 5 g/dl), or hypoglycaemia (glycaemia < 2.2 mmol/ml), and 17 with severe cerebral malaria (CM) with a Blantyre Coma Score < 2, or three convulsive episodes during 24 hours before admission with post-critical comatose > 15 minutes]. Two control groups were recruited: an uninfected group, also called endemic control (EC) group, comprising 17 children with *P. falciparum*-negative thin blood smear, and asymptomatic infected group (AI) comprising 22 children with no clinical manifestation of malaria but a *P. falciparum*-positive thin blood smear.

Patients from India

Malaria patients were recruited in the village of Gondia, an endemic region in the north-east of the Maharashtra State of India. The village is surrounded by forest. Gondia has been known as an endemic area for at least the last 20 years. *P. falciparum* appeared in Gondia over the last 10 years. It is transmitted during the rainy season in June,

Table 1: Characteristics of both studied cohorts: Gabon and India. Clinical group description, according to the number of patients, age, sex and parasitaemia.

	Clinical Groups	Staff	**Age-mean** (min-max)	**Sex** (male-female)	**Parasitemia** (%)
Gabon	**EC**	17	2,7 (0,5–5)	10-7	-
	AI	22	2,9 (0,1–5)	12-10	0.22 (0.01–1.2)
	UM	50	3 (0,5–5)	22–28	6.88 (0.05–48.6)
	SM	29	1,8 (0,2–4,5)	18-11	5.91 (0.08–31)
	CM	17	2,4 (0,5–5)	12-5	10.42 (0.15–64)
	Total	145	2,6 (0,1–5)	74-61	
Índia	**NEC**	9	32,7 (25–63)	8-1	-
	EC	14	27,3 (23–37)	13-1	-
	UM	31	30,8 (4–70)	18-13	1,24 (0,71–2,18)
	SM	13	30,4 (8–65)	10-3	1,11 (0,38–3,21)
	CM	26	40,4 (9–72)	16-10	2,04 (1,07–3,87)
	ExCM	5	19,36 (7–51)	0–5	-
	Total	93	32,3 (4–72)	65-28	1,50 (1,02–2,20)

NEC- non endemic control, EC- endemic control, AI- asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria, ExCM – Ex-cerebral malaria. Note: sex couldn't be determined for 1/18, 11/61 and 4/33 patients from EC, UM and SM, respectively.

peaks in the winter season (November, December and January) and becomes rare as summer approaches (March, April and May). The studied groups consisted of 98 patients from four to 72 years of age, being predominantly adults (see Table 1). Six cohorts were constituted according to WHO criteria for uncomplicated and severe malaria: two control groups of uninfected individuals from non-endemic (NEC) and endemic regions (EC) comprising nine and 14 patients respectively; three groups of infected patients, with 31 developing uncomplicated malaria (UM), 13 developing severe non-cerebral malaria (SM) and 26 developing cerebral malaria (CM); and one group of five patients that had recovered from CM (ex-cerebral malaria patients, Ex-CM). Between eight and 10% of the CM malaria cases died. UM cases were treated as outpatients. SM patients were admitted to hospital fully conscious and could respond well verbally to doctors' questions. CM cases were in coma. Drug treatment was paracetamol, quinine and arteether (E-mal®). Samples were collected after obtaining the consent of the patients, or of their families. Blood samples from endemic controls were collected from the relatives of malaria patients (brothers/sisters/parents) with their consent. These controls had not suffered from malaria during the previous two years. Non-endemic blood samples were collected from individuals who had not suffered from malaria during the previous five years.

Blood sample collection and parasite assessment

Venous blood was collected on EDTA in sterile vacutainers from each patient on the day of hospitalization (day 0, before any treatment), and seven (day 7) and thirty days later (day 30). Plasma was obtained by centrifuging the blood samples at 5000 rpm for 15 min. Plasma samples were stored at -80°C until use.

Parasitaemia was assessed by counting asexual forms of *P. falciparum* on thin blood smears under a light microscope after Giemsa staining. Parasitaemia was expressed as the mean percentage of infected red blood cells.

Culture of malaria parasites

Erythrocytic stages of the *P. falciparum* malaria parasite line *FAN 5HS* (source: NCCS, Pune, India) and 3D7 were cultured using candle jar dessicators as previously described [19]. The culture medium was RPMI 1640 (Gibco-BRL), supplemented with 0.5% AlbuMix (Gibco BRL). The cultures were maintained in six-well or 24-well tissue culture plates (NUNC). Parasitaemia was 5% at the start of culture and reached 25% after six days. Culture medium and fresh RBCs were added every other day.

Preparation of parasite extracts

Parasite soluble antigen was prepared from synchronous cultures containing more than 20% mature trophozoites; more than 6% rings and more than 5% schizonts were used. The cultures were pooled and centrifuged at 3,000 rpm at 4°C, and the pRBC pellet was kept and the supernatant discarded. The pRBC pellet was suspended in 10 ml sterile PBS 1 × (0.15 M, pH7.2) and then centrifuged. The parasitized red blood cell (pRBC) pellet was washed five times and then lysed by adding 15 ml of 0.1% saponin. The saponin treatment frees the parasites from the infected RBCs. This was centrifuged at 6,000 rpm for 30 min at 4°C. The supernatant was discarded and the parasite pellet was washed five or six times with sterile

cold PBS. The parasite pellet was resuspended in 1 ml protein isolation buffer containing a cocktail of protease inhibitors. This was briefly sonicated and the tube was kept at 4°C for between four and five hours. The contents of the tube were agitated by cyclo-mixing and then centrifuged at 6,000 rpm for 30 min at 4°C. The clean supernatant was collected in a separate tube and the pellet was discarded. The contents were sterilized by passing through 0.22 μm-pore filters. Aliquots of the antigen were frozen at -70°C until use. Parasite proteins were quantified by the Bradford method. The concentration of the parasite line FAN 5HS and 3D7 were 1.2 and 2.6 mg/ml, respectively.

Normal RBC extracts

Normal red blood cell (RBC) extract was prepared from the same batch of RBCs used for culturing the parasites, and followed the same procedure as previously described for pRBCs. Briefly, the RBCs were washed with PBS and the buffy coat was removed. After centrifugation, the RBC pellet was suspended in 1 ml protein isolation buffer containing a cocktail of protease inhibitors. This was briefly sonicated and the tube was kept at 4°C for between four and five hours. The contents of the tube were agitated by cyclo-mixing and then centrifuged at 6000 rpm for 30 min at 4°C. The clean supernatant was collected in a separate tube and the pellet discarded. The protein contents were estimated using a protein determination kit (BCATM protein assay Kit, Pierce, France).

Total IgE levels

An ELISA method was used to detect total IgE plasma levels in samples corresponding to day 0, day 7 and day 30. ELISA plates (96 microwell plates, reacti-bind 96 EIA Plate 100/PKG, Pierce) were coated with 50 μl/well of purified sheep polyclonal anti-human IgE solution at 5 μg/ml (The Binding Site, Birmingham UK) by incubation overnight at 4°C. The plasma samples were diluted 1:5 and incubated for two hours at 37°C. Bound IgE was detected using a peroxydase-conjugated polyclonal anti-human-IgE (The Binding Site, Birmingham UK). Binding was revealed using the OPD substrate (Sigma) and the product was quantified from the optical density (OD) at 450 nm. Serial dilutions ranging from 2 μg/ml to 0.0019 μg/ml of IgE solution (human monoclonal IgE provided by Dr Thierry Batard – Stallergenes, Anthony, France) gave the standard curve. The median of each optical density value was fitted into the sigmoidal standard curve using a specific ELISA programme running in Igor version 3.16 (Wavemetrics, Lake Oswego, OR).

IgE functional assay

A new rat mast cell line RBL-2H3 transfected with a human Fcε RI α-chain that triggers degranulation upon human IgE cross-linking was used [20]. Cells were maintained in Dulbecco medium (Gibco BRL, Eragny, France) containing 10% foetal bovine serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin (GIBCO BRL, France). Cells were expanded by incubation at 37°C for three to four days in complete Dulbecco medium supplemented with G418 (GIBCO BRL, France).

β-Hexosaminidase is known as a component of the basophil and the mast cell specific granule, and is released during degranulation of these cells [21]. Degranulation was monitored after antigen stimulation by measuring the level of released β-hexosaminidase. Fcε RI α-chain RBL-2H3 transfected rat mast cell line cultures (5×10^5 cells per well) were incubated with the different serum samples at a non-cytotoxic dilution (previously determined) for 48 hours at 37°C in the absence of the G418 antibiotic. The upregulated receptors were saturated by incubation at 4°C for 30 minutes with the same samples diluted 1:10. The cells were then washed with PBS 1X, centrifuged and resuspended in 1 ml Tyrode buffer before being centrifuged again. Finally, the cell pellet was resuspended in 450 μl of D2O (50%) and Tyrode buffer (50%) solution and each culture sample was distributed to 10 ELISA plate wells. Different controls were carried out for each sample. Control cells on lane 1 and 2 were subjected to Triton disruption (Triton 5%) and represented 100% enzyme release. Cells on lanes 3 and 4 were incubated with 50 μl of complemented Dulbecco medium without serum and represented the background enzyme release. Lanes 5 and 6, 7 and 8, 9 and 10 were incubated with 50 μl of different duplicated concentrations of parasite extract (1,000, 100 and 10 ng/ml) for 30 minutes at 37°C. After centrifugation of each well sample, 50 μl of each supernatant was collected and incubated with 50 μl of PNAG substrate solution for 90 minutes at 37°C. The level of released β-hexosaminidase was estimated from the OD at 405 nm using a spectrophotometer. All results are expressed as the percentage of total β-hexosaminidase in the cells after correcting for spontaneous release in unstimulated cultures, calculated as following: (experimental β-hexosaminidase – background β-hexosaminidase)/(total β-hexosaminidase – background β-hexosaminidase) × 100.

Flow cytofluorometry analysis

FACS analysis was performed after incubating RBL-2H3-D12.8 cells with several dilutions of serum samples to follow the induction of the high affinity receptor (Fcε RI) expression after stimulation by IgEs in the patient's sera. Cells were incubated for 48 hours at 37°C with the different serum samples optimally diluted to avoid cytotoxicity. A saturation step with the same sera diluted 1:10 was done by incubation at 4°C for 30 minutes. Cells were washed with PBS 1X and incubated with FITC-labelled anti-IgE (Tebu, Le Perray en Yvelines, France) (1/100) for 30 minutes. Cells were washed again, centrifuged, resuspended in

PBS 1X and analysed by cytofluorometry using Cellquest software (Beckton Dickinson, USA). 10,000 cells were acquired per tube.

Cytokine levels

The levels of cytokines in the plasma (IL-4, TNF, INF-γ, and IL-10) were estimated by Opti-ELISA kits (Pharmingen, San Diego, CA.USA) used following the manufacturer's instructions.

Statistics

Due to a non-normal distribution of the scores in each group, non-parametric tests were performed, using the median to compare the different clinical groups. The Mann Whitney test was used for comparisons between two groups and the Kruskall Wallis test to compare three or more groups. Spearman's correlation was used to check for correlations between parameters. P values less than 0.05 were considered as significant. Chi-squared test was used to compare qualitative variables.

Results

Serum total IgE levels in groups of P. falciparum *infected patients with different clinical phenotypes*

Total IgE levels were analysed in endemic controls and in cohorts of *P. falciparum*-infected patients with different clinical forms of malaria, ranging from asymptomatic to cerebral disease, from Gabonese and Indian endemic areas to study the association between the IgE response and disease severity. Total IgE levels were measured by ELISA in individual sera before drug administration (corresponding to day 0) and determined the general distribution in the studied populations from Gabon (Figure 1A) and India (Figure 1B). Total IgE concentrations were found to be much higher in patients from India (mainly adults) than in patients from Gabon (children). In both populations independent of the different levels of IgE in each population, the median IgE levels within each clinical group tended to increase upon infection (mainly in UM and SM groups), although the difference between the groups was only significant in the Indian population (Kruskall Wallis, p = 0.0005). As only Indian patients showed a significant difference, the Mann Whitney test was used to compare the different groups in this population only. There was a significant increase in IgE levels in the EC group compared to the NEC group (p = 0.042). The most significant increase in IgE levels (versus the EC group) occurred in the UM patients (p = 0.015) and in the SM patients (p = 0.013). No significant difference between the EC group and the CM and Ex-CM groups was observed.

A range of values of IgE levels was defined enabling the analysis of the frequency of normal, moderate and high IgE levels in each clinical group of patients. The so-called normal values were adjusted to the studied population because the Gabonese and Indian groups had different plasma total IgE ranges. Therefore, the normal value (N) was defined by the median IgE levels in the endemic controls of each study population. Consequently, all values between N and 2N were considered as low/moderate IgE levels and those between 2N and 3N as moderate/high IgE levels, with the highest levels being above 3N (Figures 1C and 1D). Even in Gabonese patients, for whom the increase of IgE in the disease groups was not significant, a higher percentage of patients with clinical disease had higher IgE levels than controls and asymptomatic patients. These differences were more marked in the UM, SM and CM Indian patients (Figure 1D). In the Indian population, the NEC group did not have moderate/high IgE levels, although a high percentage of patients exhibited normal IgE levels (Figure 1D). Also, no significant change was detected in IgE levels over time in the UM, SM and CM groups of the Gabonese cohorts when tested seven days and 30 days after treatment (Table 2). No significant association of malaria and IgE levels with sex in the two studied populations. However, a significant increase in IgE levels with age (p = 0.00034) was observed in the Gabonese subjects (Figure 2A) but not in the Indian subjects.

The correlation between IgE levels and the parasite load was tested. Although the general trend was different in the Indian and Gabonese population, there was no significant correlation between IgE concentration and parasite load for all groups together. In Gabonese cohorts, a negative correlation for all groups was observed, except for the UM patients where the correlation showed a positive tendency. In the Indian cohorts, a positive correlation was observed between IgE levels and parasite load, mainly in the UM and SM groups (Figure 2B).

Functional parasite specific IgE response in P. falciparum *infected patients*

Previous studies have used ELISA to quantify specific IgE present in the serum [7,8,10,11]. The functionality of specific IgEs present in the serum was studied by evaluating the ability of these IgEs to induce mast cell degranulation in the presence of the parasite antigen. A rat mast cell line transfected with the human α-chain of Fcε RI was used [20]. Human Fcε RI expression was induced after incubation with all serum samples at non-cytotoxic dilutions. FACS was used to detect the presence of FcåRI receptors on the mast cells surface induced by IgE present in serum samples. Although the fluorescence intensity revealing human Fcε RI expression by the mast cells varied between patient samples, IgE receptors were upregulated in all the samples tested. No correlation between total IgE levels in the serum and the up-regulation of mast cell receptors was found. There was no significant correlation between IgE

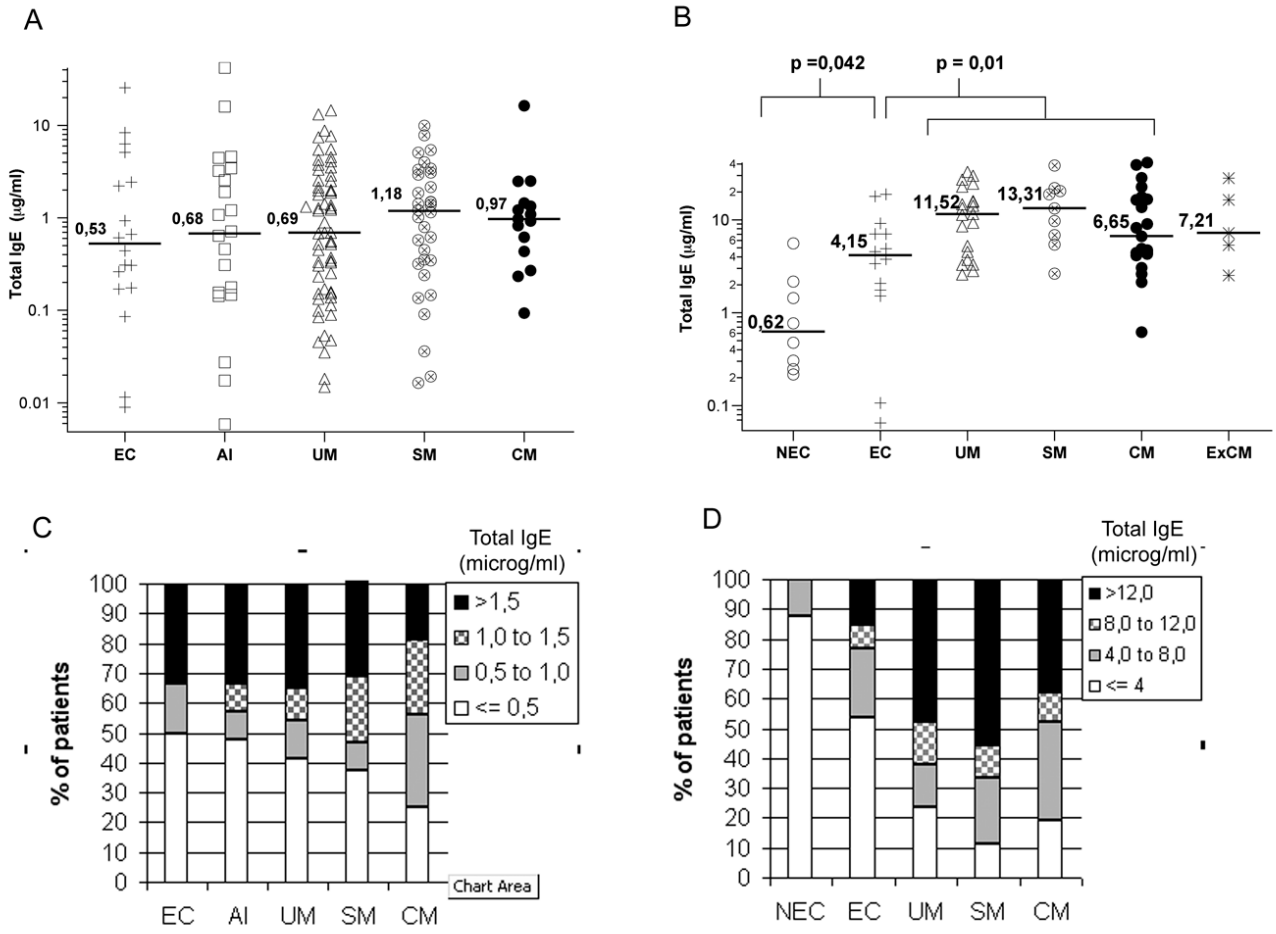


Figure 1

Distribution of total IgE levels per clinical group in both studied populations: Gabon and India. **A.** Total IgE levels (µg/ml) per clinical group in Gabonese patients (non-significant Kruskall Wallis test). **B**. Total IgE levels (µg/ml) in Indian patients (significant Kruskall Wallis test, p = 0.0005). **C.** Percentage of patients with defined IgE levels per group in the Gabonese population (normal levels (N) lower than or equal to 0.500 µg/ml, moderate levels (N to 2N), from 0.501 to 1.000 µg/ml, high levels (2N to 3N), from 1,000 to 1,500 µg/ml, very high (>3N) greater than 1,500 µg/ml). **D.** Percentage of patients with defined levels of IgE per group in the Indian population (normal levels (N) lower than or equal to 4,000 µg/ml, moderate levels (N to 2N), from 4,000 to 8,000 µg/ml, high levels (2N to 3N), from 8,000 to 12,000 µg/ml, very high (>3N), greater than 12,000 µg/ml). Legend: EC – endemic control, AI – Asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria, CM – cerebral malaria, NEC – non-endemic control, ExCM – ex-cerebral malaria.

levels and receptor upregulation and the level of mast cell degranulation. Sensitized cells were then incubated with different concentrations of a *P. falciparum* blood-stage antigen extract. Mast cell degranulation was measured by quantifying the release of β-hexosaminidase. Control cells not exposed to any serum gave a maximum mast cell degranulation of 3%. Therefore, serum samples giving an enzyme release greater than 5% in the presence of at least one of the antigen concentrations were considered positive for functional IgE. In the Gabonese cohorts (Figure 3A), there were functional *P. falciparum* IgE in all clinical groups. However, the percentage of patients with functional specific anti-parasite IgE was higher in asymptomatic and uncomplicated malaria patients than in other groups. Also, the percentage of patients displaying parasite-specific IgE was lower in the group exhibiting severe disease. The distribution of patients per group releasing between 5 and 10%, 10 and 30% and above 30% β-hexosaminidase induced by specific anti-parasite IgE revealed that one patient in CM group had a degranulation level above 30%, being the highest induced response among all the tested individuals. The same assay was carried out on

Table 2: Day 0, day 7 and day 30 median IgE levels per clinical group in the Gabonese population.

Total IgE (μg/ml)	EC (min-max)	AI (min-max)	UM (min-max)	SM (min-max)	CM (min-max)
Day 0	0,525 (0,009–25,47)	0,677 (0–41,96)	0,690 (0,015–13,173)	1,132 (0,016–9,915)	0,922 (0,093–16,34)
Day 7	0,582 (0,0144–5,644)	0,448 (0,0192–3,188)	0,516 (0,011–5,228)	0,836 (0,016–5,689)	8,033 (8,033–8,033)
Day 30	1,026 (0,044–2,628)	0,977 (0,056–7,446)	0,151 (0,052–3,925)	0,662 (0,011–6,315)	------

EC – endemic control, AI – asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria.

the Indian population. Although there was no significant difference between groups, the percentage of patients having functional IgE recognizing the parasite extract was slightly higher in EC and UM groups than in SM and CM groups (Figure 3B). All positive patients had an enzyme release of between 10 and 30%. No significant correlation was found between *P. falciparum*-specific IgE-induced mast cell degranulation levels and sex, age and parasitaemia.

Relationship between total and specific serum IgE and cytokine profiles

IgE production is influenced by cytokines produced by activated T cells. These cytokines are also involved in pathophysiological mechanisms associated with severe malaria [4,22,23]. Therefore, the relationship between the cytokine profile, the IgE levels and the clinical manifestation was investigated. IFN-γ, TNF and IL-10 levels were measured in the sera of the Indian and Gabonese patients. IL-4 levels were measured only in Indian patients as it was not different between the Gabonese groups. IFNγ concentrations were highest in the Gabonese AI and CM groups (Table 3). This cytokine is significantly higher in the AI and CM groups than in EC ($p = 0.02$ and $p = 0.009$ respectively). The plasma TNF concentration was similar in the severe SM and CM groups and in disease-free EC and AI groups. TNF levels were clearly higher in SM and CM groups than in the UM group ($p < 0.001$). Surprisingly, EC and AI also exhibited higher TNF levels when compared to UM group. No association between IFN-γ or TNF and IgE levels were found. However, a significant positive correlation was found between the concentration of total IgE and IL-10 in the UM group ($p = 0.02$) and a significant negative correlation in the AI group ($p = 0.02$) (Figure 4). The median levels of the different cytokines in the plasma of Indian patients are given in Table 4. IL-10 and TNF levels were higher in CM patients than in controls and other *P. falciparum*-infected patients. The plasma concentrations of these cytokines were moderate in cured CM patients (ExCM). Their levels of IL-10 and TNF were slightly higher in endemic controls than in non-endemic controls. Levels of IFN- were lower in the CM group than in AI group. No

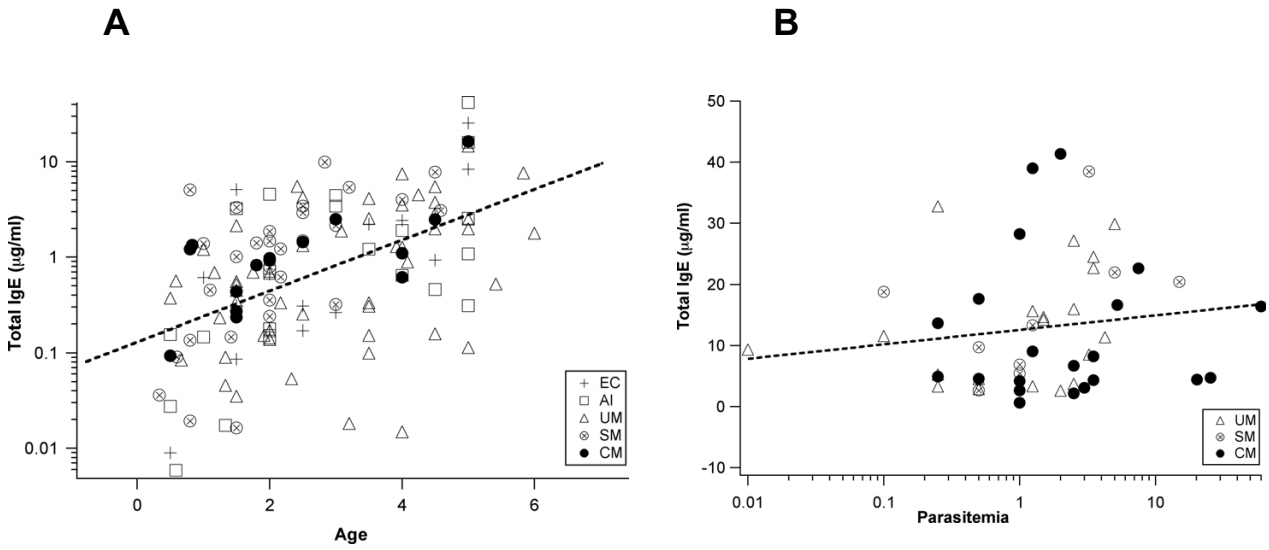


Figure 2
A. Total IgE correlation with age in the Gabonese population (significant spearman correlation, p = 1.0 × 10-9). **B.** Total IgE correlation with parasitaemia in the Indian population. Significant Spearman correlation (p = 0,0001).

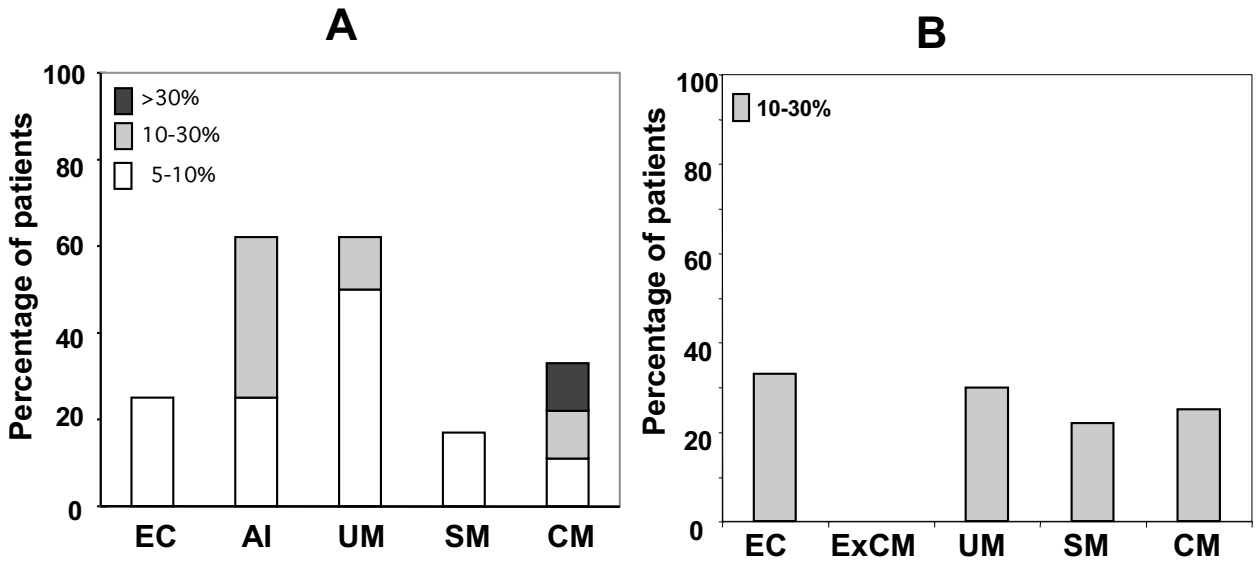


Figure 3
Percentage of patients with positive functional IgE against parasite antigen in the Gabonese and Indian populations. **A.** Distribution of patients with positive anti-parasite functional IgE, exhibiting different intensities of enzyme release per clinical group in the Gabonese population (low enzyme release, from 5 to 10%; moderate enzyme release, from 10 to 30%; and high enzyme release, greater than than 30%). **B.** Distribution of patients with positive anti-parasite functional IgE in the Indian population.

difference was found for the levels of IFN-γ between the uncomplicated and severe disease. Although, there was a significant correlation between IgE levels and IFN-γ (Figure 5A), TNF (Figure 5B) and IL-10 (Figure 5C) levels when looking at all the groups combined. Most diseased groups had high cytokine levels, whereas control groups had lower levels (Figure 5A, B and 5C).

Discussion

The main feature of this study is the comparison of total and functional *P. falciparum*-specific IgE responses in two populations of low and high malaria transmission levels, India and Gabon respectively and their relationship with disease severity. Several clinical groups were compared: endemic non-infected controls, asymptomatics and different clinical manifestations including uncomplicated, severe non-cerebral and cerebral malaria.

Indian and Gabonese individuals exhibited a different range of plasma levels of circulating IgE These cohorts had a different age range, with the Gabonese groups being children from 0 to 6 years of age, whereas Indian patients had a mean age of 30 years. In the two populations, irrespective of its concentration and also consistent with previously published data, the IgE distribution tended to increase upon parasite stimulation [8,10,11]. In the Indian population, circulating IgE levels were seven times higher in endemic controls than in non-endemic controls. This suggests that exposure to the parasite strongly influences the production of IgE, although this difference may also be due to other endemic factors [7]. Nevertheless, it was reported that IgE levels were greatest in patients developing severe disease than in CM group (Figure 1A and 1B). When considering the percentage of patients that produce high levels of IgE per group, it was shown that IgE

Table 3: Cytokine distribution in the Gabonese population: median TNF, IFN-γ and IL-10 levels per clinical group.

	EC (min-max)	AI (min-max)	UM (min-max)	SM (min-max)	CM (min-max)
TNF (pg/ml)	41 (8–94)	36 (0–395)	8 (0–440)	175 (1–442)	209 (0–1520)
IFN-γ (pg/ml)	3,2 (0–8)	9,5 (0–31)	5 (0–395)	4 (0–201)	6,2 (2,5–9)
IL-10 (pg/ml)	14 (0–83)	102 (0–317)	134 (0–1380)	339 (0–5200)	95 (0–2300)

EC – endemic control, AI – asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria.
Comparing group by group with the Endemic control: TNF was significantly lower in UM (p = 0,0006); IFN-γ was significantly higher in AI and CM (p = 0,02 and p = 0,007, respectively); IL-10 was significantly higher in UM (p = 0,0009), SM (p = 0,0001) and CM (p = 0,0001)

Table 4: Cytokine distribution in the Indian population: median TNF, IFN-γ and IL-10 per clinical group.

	NEC (min-max)	EC (min-max)	UM (min-max)	SM (min-max)	CM (min-max)	ExCM (min-max)
TNF (pg/ml)	57 (39–73)	78 (63–120)	180 (119–207)	200 (173–381)	530 (258–1227)	81 (69–124)
IFN-γ (pg/ml)	23 (10–31)	22 (17–201)	119 (70–153)	127 (111–200)	65 (45–101)	32 (11–53)
IL-10 (pg/ml)	13 (5–26)	22 (11–31)	120 (97–147)	176 (121–253)	301 (175–506)	85 (40–108)
IL-4 (pg/ml)	18 (8–40)	58 (23–84)	65 (31–204)	69 (50–89)	62 (40–85)	48 (29–80)

NEC – non endemic control, EC – endemic control, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria, ExCM – Ex-cerebral malaria.
Comparing group by group with the Endemic control: TNF was significantly lower in NEC (p = 0,0006) and higher in UM (p = 1,19 × 10-7), SM (p = 1,01 × 10-5) and CM (p = 2,26 × 10-7); IFN-γ was significantly higher in all diseased groups (UM – p = 5,11 × 10-6, SM – p = 1,01 × 10-6, CM – p = 9,71 × 10-6); IL-10 was significantly higher in all diseased groups (UM – p = 1,04 × 10-7, SM – p = 1,01 × 10-5 and CM – p = 2,46 × 10-7).

levels are higher in UM and SM (Figure 1C and 1D) than in CM patients who had values similar to that of the controls [8,24]. In addition, the median levels of circulating IgE in the ExCM group were close to that of the CM group. Also, no significant correlation was found between both IgE and TNF levels in the CM group. These observations are contrary to published data describing increased IgE levels that correlate with high concentrations of circulating TNF, a cytokine associated with malaria severity and also with pRBC adherence on brain capillary endothelial cells [9,10,25,26]. The results suggest that either IgE does not play an important role in CM pathogenesis, or that these antibodies may participate in the parasite sequestration into the brain or other organ capillaries [24].

There was a significant increase in IgE levels with age in Gabonese children independent of the disease group (Figure 2). This increase in IgE production between 0 and six years of age may also reflect an increase in the capacity of the immune system to respond to parasite infections [7,27]. Such a correlation was not found in Indian groups. This would be expected because the Indian groups comprised mainly adults, with the few children being older than five years. Although most of individuals in the Gabonese endemic control group had already been in contact with the parasite, as demonstrated by the high titres of specific antibody to *P. falciparum*-infected red blood cells observed in these children, the median plasma IgE concentrations were compared with those of the Indian NEC group. Although the median total IgE levels in the Indian NEC groups was higher than that of the Gabonese EC group, suggesting that age could be an important factor in IgE production, the higher IgE levels in the Indian population may also be interpreted as the result of either environmental factors, such as predominance of food allergies, of the genetic background, which may predispose to developing IgE responses [13,27,29-32] or of a co-infection with other parasites, such as helminths affecting the IgE responses in these patients [33,34]. In the Gabonese cohorts, IgE levels tended to correlate negatively to parasitaemia except in UM whereas all patient groups from India showed a positive correlation.

The pool of circulating IgE comprises both monomeric and complexed immunoglobulins [35,36]. A functional test was performed, based on the ability of the circulating IgE from the sera of different patient groups to induce degranulation of mast cells in the presence of pRBC antigens to better estimate the *P. falciparum* specific IgE response. This test does not provide specific IgE concentration within total IgE. It is based on the specific IgE induced percentage of mast cells degranulation. Degranulation was measured by quantifying β-hexosaminidase release. Functional *P. falciparum*-specific IgEs were detected in randomly chosen patients from all groups in both the Gabonese and Indian populations, except for ExCM Indian patients. The highest percentage of patients with functional anti-parasite IgEs was found in the Gabonese AI and UM and Indian EC and UM groups, which decreased in the SM and CM groups (Figure 3A and 3B). This suggests a protective role for *P. falciparum*-specific IgE, and is consistent with previous published data [13,14].

Although the CM group had a low percentage of patients able to induce degranulation, it was the only group where there was one patient serum inducing a mast cell degranulation above 30%. This intense response may be associated with the presence of IgE with higher affinity for *P. falciparum* antigens, as previously reported by Gonzalez-Espinosa *et al.* [37]. However, the level of degranulation can also be enhanced by the number of receptors involved in recognizing the antigen-IgE complex, which can strongly affect the size of the secretory response [37,38]. There was no evident correlation between the level of functional *P. falciparum*-specific IgE (percentage of enzyme release) and the level of total IgE per group within each population. This is unsurprising, given that both the monomeric and complexed forms of circulating parasite-specific IgE can affect the level of degranulation [39-41]. It

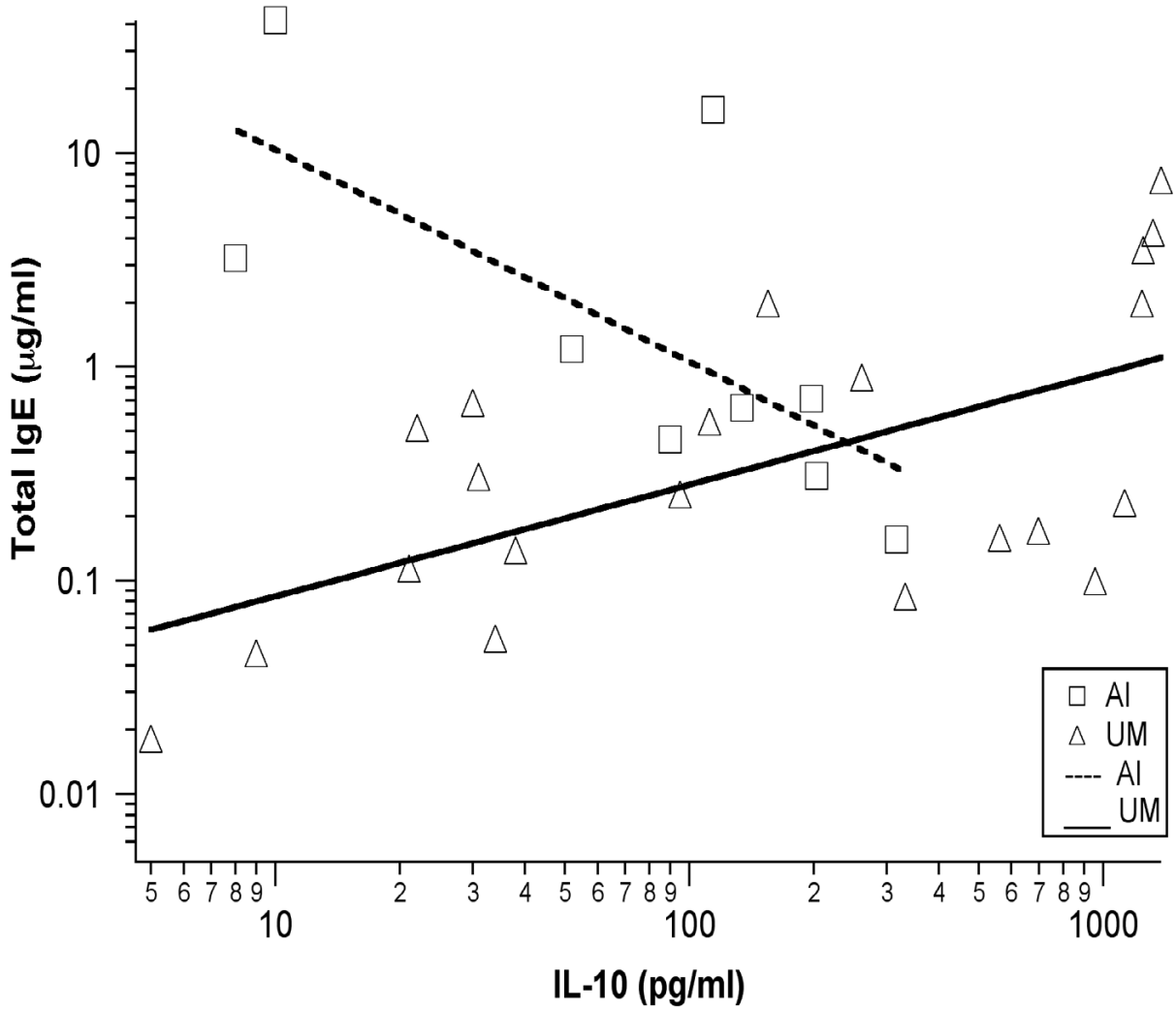


Figure 4
IL-10 correlation with total IgE levels in the Gabonese population. **Dashed line -** Asymptomatic patients (significant negative spearman correlation, p = 0.025). **Bold line -** Uncomplicated malaria patients (significant positive correlation, p = 0.017).

is also acceptable that total IgE levels were not directly correlated to FcåRI upregulation levels [40,42]. The minimum level of receptors occupied by the parasite antigen-specific IgE complex required to induce a degranulation response is 10%. Consequently, this response will be independent of the total IgE levels [20]. An increase in specific IgE levels has been also seen in other parasitic infections, such as helminthiasis, in which the specific IgEs usually help to eliminate the pathogens either through hypersensitivity reactions resulting from mast cell degranulation or by inducing antibody-dependent cell-mediated responses [27].

High levels of IgE in *P. falciparum*-infected individuals have been shown to be due to an underlying imbalance in favour of IL-4 in the ratio of $CD4^+$ T cell producers, which are responsible for the IgG/IgM isotype switching to IgE [10]. High levels of circulating IL-4 have also been associated with a greater parasite antigen-specific production of IgE in individuals less susceptible to malaria [13]. Also, Th1-type pro-inflammatory cytokines, such as IFN-γ and TNF are thought to play an important role in the both protecting against and increasing the pathogenesis of cerebral malaria [3,4,22]. Plasma TNF, IFN-γ and IL-10 levels were measured in both the Indian and Gabonese groups. In the

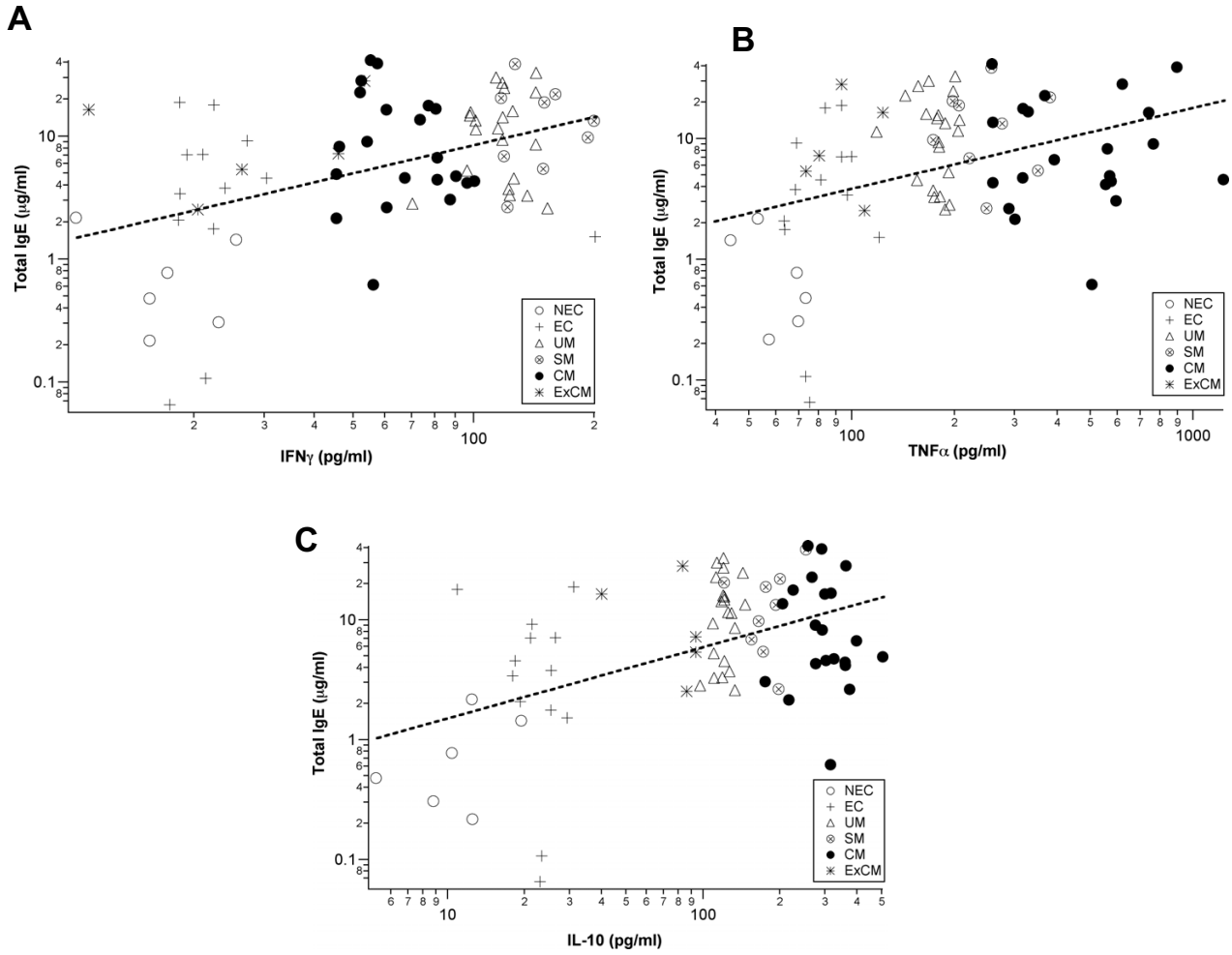


Figure 5
Cytokine correlation with IgE levels in the Indian population. **A.** TNF correlation with total IgE levels (significant positive spearman correlation, p = 0.0037); **B.** IFN-γ correlation with total IgE levels (Significant positive spearman correlation, p = 0.0028); **C**. IL-10 correlation with total IgE levels (significant positive spearman correlation, p = 0.0051).

Gabonese groups, no direct correlation was found between IgE and IFN-γ or TNF levels in the UM, SM and CM patients. However, the level of IgE production was correlated to IL-10 levels in UM and AI patients (Figure 4). In this group, there was a positive association between IL-10 levels and parasite load, but not between IgE levels and parasitaemia. In the Indian groups, IFN-γ, TNF and IL-10 levels were all significantly correlated with IgE levels independent of the group (Figures 5A, B and 5C). When looking for a correlation per group, no significant statistic value was found. IL-4 is the main cytokine responsible for IgE production, inducing antibody isotype switching from IgG and IgM to IgE [10]. Previous studies have shown an association between the IL-4/IFN-γ levels and IgE levels, suggesting an induced Th2-type switched response [43]. As IL-4 levels were different between groups only in Indians, a correlation was expected between this ratio and IgE levels, and surprisingly, the results showed an opposite correlation (p = 0,012). The levels of IL-4 remained almost constant across all groups as IgE levels increased. Therefore, this opposite correlation arises due to a parallel increase in both IFN-γ and IgE levels. This suggests that, in the Indian population, IL-4 does not seem to directly influence IgE levels. It has been shown experimentally that there are conditions under which alternative mechanisms may induce IgE production independent of IL-4 [44]. Regarding other cytokine ratios, like TNF/IL-10, no significant correlation was found with total IgE levels.

In conclusion, these results showed that total IgE levels increased in infected patients, mainly in UM and SM patients but not in CM patients. The decrease in total IgE levels in the CM group was associated with a higher intensity of mast cell degranulation induced *in vitro* by *P. falciparum*-specific IgE. In addition, no correlation was found between total and functional *P. falciparum*-specific IgE levels.

The results reported here show the high activity of functional circulating *P. falciparum*-specific IgE in asymptomatic malaria patients. However, no correlation was observed between plasma levels of total IgE, the disease phenotype and the cytokines pattern in the different groups of patients studied. The opposite association between pro- and anti-inflammatory cytokine ratios and IgE levels reveals the complexity of immune response disruption occurring during malaria in patients from low and high malaria endemic region.

Authors' contributions

Joana Duarte contributed to the acquisition, analysis, interpretation of data and manuscript drafting but not the design of the study. Prakash Deshpande and Vincent Guiyedi have highly contributed to conception and interpretation of data and manuscript revising. Salah Mécheri collaborated to the design and interpretation of IgE functional tests and manuscript drafting. Constantin Fesel contributed to the statistical analysis, interpretation of data and manuscript drafting.

Pierre-André Cazenave, Gyan C. Mishra and Maryvonne Kombila have contributed to conception of the study and manuscript revising. Sylviane Pied participated to conception, design, analysis and interpretation of data, the final approval of the version to be published.

Financial support

This work was part of the *Centre National de la Recherche Scientifique-Laboratoires Européens Associés* "*Génétique et developpement de la tolérance naturelle*" program. It was supported by the PAL+ program of the French Ministry of Research. We thank the Indo-French Centre for the Promotion of Advanced Research (IFCPAR), New Delhi, India for providing financial assistance (project No.2103-3). C-F received a post-doctoral fellowship from the *Fundaçâo para a Ciência e Tecnologia* (Portugal). V-G holds a fellowship from the *Agence Universitaire de la Francophonie* (AUF).

Acknowledgements

We thank M. Bouyou-Akotet and M. Idrissa-Boubou for assistance with the collection of plasma samples. We are grateful to R.O. for critical reading of and his help in preparing the manuscript.

References

1. Moorthy VS, Good MF, Hill AV: **Malaria vaccine developments.** *Lancet* 2004, **363:**150-156.
2. Miller LH, Baruch DI, Marsh K, Doumbo OK: **The pathogenic basis of malaria.** *Nature* 2002, **415:**673-679.
3. Heddini A: **Malaria pathogenesis: a jigsaw with an increasing number of pieces.** *Int J Parasitol* 2002, **32:**1587-1598.
4. de Souza JB, Riley EM: **Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis.** *Microbes Infect* 2002, **4:**291-300.
5. Adams S, Brown H, Turner G: **Breaking down the blood-brain barrier: signaling a path to cerebral malaria?** *Trends Parasitol* 2002, **18:**360-366.
6. Miller LH, Good MF, Milon G: **Malaria pathogenesis.** *Science* 1994, **264:**1878-1883.
7. Calissano C, Modiano D, Sirima BS, Konate A, Sanou I, Sawadogo A, Perlmann H, Troye-Blomberg M, Perlmann P: **IgE antibodies to *P. falciparum* and severity of malaria in children of one ethnic group living in Burkina Faso.** *Am J Trop Med Hyg* 2003, **69:**31-35.
8. Perlmann H, Helmby H, Hagstedt M, Carlson J, Larsson PH, Troye-Blomberg M, Perlmann P: **IgE elevation and IgE anti-malarial antibodies in *P. falciparum* malaria: association of high IgE levels with cerebral malaria.** *Clin Exp Immunol* 1994, **97:**284-292.
9. Perlmann P, Perlmann H, ElGhazali G, Blomberg MT: **IgE and tumor necrosis factor in malaria infection.** *Immunol Lett* 1999, **65:**29-33.
10. Perlmann P, Perlmann H, Flyg BW, Hagstedt M, Elghazali G, Worku S, Fernandez V, Rutta AS, Troye-Blomberg M: **Immunoglobulin E, a pathogenic factor in *P. falciparum* malaria.** *Infect Immun* 1997, **65:**116-121.
11. Perlmann P, Perlmann H, Looareesuwan S, Krudsood S, Kano S, Matsumoto Y, Brittenham G, Troye-Blomberg M, Aikawa M: **Contrasting functions of IgG and IgE antimalarial antibodies in uncomplicated and severe *P. falciparum* malaria.** *Am J Trop Med Hyg* 2000, **62:**373-377.
12. Seka-Seka J, Brouh Y, Yapo-Crezoit AC, Atseye NH: **The role of serum immunoglobulin E in the pathogenesis of *P. falciparum* malaria in Ivorian children.** *Scand J Immunol* 2004, **59:**228-230.
13. Bereczky S, Montgomery SM, Troye-Blomberg M, Rooth I, Shaw MA, Farnert A: **Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria.** *Int J Parasitol* 2004, **34:**935-942.
14. Farouk SE, Dolo A, Bereczky S, Kouriba B, Maiga B, Farnert A, Perlmann H, Hayano M, Montgomery SM, Doumbo OK, Troye-Blomberg M: **Different antibody- and cytokine-mediated responses to *P. falciparum* parasite in two sympatric ethnic tribes living in Mali.** *Microbes Infect* 2005, **7:**110-117.
15. Oettgen HC, Geha RS: **IgE regulation and roles in asthma pathogenesis.** *J Allergy Clin Immunol* 2001, **107:**429-440.
16. Nyakeriga MA, Troye-Blomberg M, Bereczky S, Perlmann H, Perlmann P, ElGhazali G: **Immunoglobulin E (IgE) containing complexes induce IL-4 production in human basophils: effect on Th1-Th2 balance in malaria.** *Acta Trop* 2003, **86:**55-62.
17. Reischl IG, Coward WR, Church MK: **Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE-FcepsilonRI) interaction.** *Biochem Pharmacol* 1999, **58:**1841-1850.
18. WHO: **Severe and complicated malaria.** *Trans R Soc Trop Med Hyg* 1990, **84:**1-65.
19. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193:**673-675.
20. Marchand F, Mecheri S, Guilloux L, Iannascoli B, Weyer A, Blank U: **Human serum IgE-mediated mast cell degranulation shows poor correlation to allergen-specific IgE content.** *Allergy* 2003, **58:**1037-1043.
21. Takagi K, Nakamura R, Teshima R, Sawada J: **Application of human Fc epsilon RI alpha-chain-transfected RBL-2H3 cells for estimation of active serum IgE.** *Biol Pharm Bull* 2003, **26:**252-255.
22. Plebanski M, Hill AV: **The immunology of malaria infection.** *Curr Opin Immunol* 2000, **12:**437-441.
23. Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G: **Role of Th1 and Th2 cytokines in immune response to uncomplicated *P. falciparum* malaria.** *Clin Diagn Lab Immunol* 2002, **9:**348-351.
24. Maeno Y, Perlmann P, Perlmann H, Kusuhara Y, Taniguchi K, Nakabayashi T, Win K, Looareesuwan S, Aikawa M: **IgE deposition in**

brain microvessels and on parasitized erythrocytes from cerebral malaria patients. *Am J Trop Med Hyg* 2000, **63:**128-132.
25. Artavanis-Tsakonas K, Tongren JE, Riley EM: **The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology.** *Clin Exp Immunol* 2003, **133:**145-152.
26. Chen Q, Schlichtherle M, Wahlgren M: **Molecular aspects of severe malaria.** *Clin Microbiol Rev* 2000, **13:**439-450.
27. Winter WE, Hardt NS, Fuhrman S: **Immunoglobulin E: importance in parasitic infections and hypersensitivity responses.** *Arch Pathol Lab Med* 2000, **124:**1382-1385.
28. Migot-Nabias F, Mombo LE, Luty AJ, Dubois B, Nabias R, Bisseye C, Millet P, Lu CY, Deloron P: **Human genetic factors related to susceptibility to mild malaria in Gabon.** *Genes Immun* 2000, **1:**435-441.
29. Poulsen LK: **Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods.** *Mol Nutr Food Res* 2004, **48:**413-423.
30. Yazdanbakhsh M, Kremsner PG, van Ree R: **Allergy, parasites, and the hygiene hypothesis.** *Science* 2002, **296:**490-494.
31. Paganotti GM, Babiker HA, Modiano D, Sirima BS, Verra F, Konate A, Ouedraogo AL, Diarra A, Mackinnon MJ, Coluzzi M, Walliker D: **Genetic complexity of *P. falciparum* in two ethnic groups of Burkina Faso with marked differences in susceptibility to malaria.** *Am J Trop Med Hyg* 2004, **71:**173-178.
32. Verra F, Luoni G, Calissano C, Troye-Blomberg M, Perlmann P, Perlmann H, Arca B, Sirima BS, Konate A, Coluzzi M, Kwiatkowski D, Modiano D: **IL4-589C/T polymorphism and IgE levels in severe malaria.** *Acta Trop* 2004, **90:**205-209.
33. Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, Vouldoukis I, Looareesuwan S: ***Ascaris lumbricoides* infection is associated with protection from cerebral malaria.** *Parasite Immunol* 2000, **22:**107-113.
34. Nacher M, Singhasivanon P, Traore B, Vannaphan S, Gay F, Chindanond D, Franetich JF, Mazier D, Looareesuwan S: **Helminth infections are associated with protection from cerebral malaria and increased nitrogen derivatives concentrations in Thailand.** *Am J Trop Med Hyg* 2002, **66:**304-309.
35. Johansson SG: **Anti-IgE antibodies in human serum.** *J Allergy Clin Immunol* 1986, **77:**555-557.
36. Marone G, Spadaro G, Palumbo C, Condorelli G: **The anti-IgE/anti-FcepsilonRIalpha autoantibody network in allergic and autoimmune diseases.** *Clin Exp Allergy* 1999, **29:**17-27.
37. Gonzalez-Espinosa C, Odom S, Olivera A, Hobson JP, Martinez ME, Oliveira-Dos-Santos A, Barra L, Spiegel S, Penninger JM, Rivera J: **Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells.** *J Exp Med* 2003, **197:**1453-1465.
38. Posner RG, Paar JM, Licht A, Pecht I, Conrad DH, Hlavacek WS: **Interaction of a monoclonal IgE-specific antibody with cell-surface IgE-Fc epsilon RI: characterization of equilibrium binding and secretory response.** *Biochemistry* 2004, **43:**11352-11360.
39. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G: **Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival.** *Immunity* 2001, **14:**801-811.
40. Kitaura J, Xiao W, Maeda-Yamamoto M, Kawakami Y, Lowell CA, Kawakami T: **Early divergence of Fc epsilon receptor I signals for receptor up-regulation and internalization from degranulation, cytokine production, and survival.** *J Immunol* 2004, **173:**4317-4323.
41. Yamaguchi M, Sayama K, Yano K, Lantz CS, Noben-Trauth N, Ra C, Costa JJ, Galli SJ: **IgE enhances Fc epsilon receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc epsilon receptor I expression and mediator release.** *J Immunol* 1999, **162:**5455-5465.
42. Saini SS, Klion AD, Holland SM, Hamilton RG, Bochner BS, Macglashan DW: **The relationship between serum IgE and surface levels of FcepsilonR on human leukocytes in various diseases: correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils.** *J Allergy Clin Immunol* 2000, **106:**514-520.
43. Elghazali G, Perlmann H, Rutta AS, Perlmann P, Troye-Blomberg M: **Elevated plasma levels of IgE in *P. falciparum* -primed individuals reflect an increased ratio of IL-4 to interferon-gamma (IFN-gamma)-producing cells.** *Clin Exp Immunol* 1997, **109:**84-89.
44. Grunewald SM, Teufel M, Erb K, Nelde A, Mohrs M, Brombacher F, Brocker EB, Sebald W, Duschl A: **Upon prolonged allergen exposure IL-4 and IL-4Ralpha knockout mice produce specific IgE leading to anaphylaxis.** *Int Arch Allergy* 2001, **125:**322-328.

Am J Physiol Lung Cell Mol Physiol 291: L512–L522, 2006.
First published March 23, 2006; doi:10.1152/ajplung.00278.2005.

Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses

Caroline L. S. George,[1] Misty L. White,[1] Katarina Kulhankova,[2] Aditya Mahajan,[1] Peter S. Thorne,[2] Jeanne M. Snyder,[3] and Joel N. Kline[2,4]

Departments of [1]Pediatrics, [2]Occupational and Environmental Health, [3]Anatomy and Cell Biology, and [4]Internal Medicine, University of Iowa, Iowa City, Iowa

Submitted 28 June 2005; accepted in final form 15 March 2006

George, Caroline L. S., Misty L. White, Katarina Kulhankova, Aditya Mahajan, Peter S. Thorne, Jeanne M. Snyder, and Joel N. Kline. Early exposure to a nonhygienic environment alters pulmonary immunity and allergic responses. *Am J Physiol Lung Cell Mol Physiol* 291: L512–L522, 2006. First published March 23, 2006; doi:10.1152/ajplung.00278.2005.—The hygiene hypothesis suggests that early life exposure to a nonhygienic environment that contains endotoxin reduces the risk of developing allergic diseases. The mechanisms underlying the hygiene hypothesis are unclear and may involve subtle immune system interactions that occur during maturation. Experimental objectives of this study were to use a novel animal model to test the hygiene hypothesis and to characterize early life immune system responses to a nonhygienic environment. Mice were reared in corn dust, a grain-processing byproduct with a high-endotoxin content and microbial products or in a low-endotoxin environment. The influence of early or later life exposure to corn dust on a subsequent allergen stimulus (ovalbumin) was assessed by bronchoalveolar lavage (BAL) cell analysis, lung histology, serum IgE, and BAL cytokine measurements. The influence of the corn dust environment on the developing pulmonary immune system was assessed by BAL cell analysis and immunostaining of lung tissue. The corn dust environment contained significantly more endotoxin ($P < 0.001$), and the dust exposures attenuated the cellular inflammatory response to ovalbumin in the adult mouse ($P < 0.01$) but did not reduce serum IgE levels or alter baseline BAL fluid proinflammatory cytokine levels. The corn dust environment did not induce significant neutrophilia in lavage fluid but significantly increased the number of antigen-presenting cells in alveolar walls early in life by ~37%. In conclusion, exposure to a nonhygienic environment did not induce significant airway neutrophilia, yet altered the population of immunologically active cells in the lung and reduced subsequent allergic inflammation.

hygiene hypothesis; immune system; development; rodent

IN RECENT DECADES, THE PREVALENCE of asthma has risen dramatically in industrialized nations, whereas inhabitants of underdeveloped countries appear to be protected from this phenomenon. The "hygiene hypothesis" (36) describes an association between the environment and reduced incidence of atopic and asthma disease. It is strengthened by epidemiological data that demonstrate an influence of early life environment on the development of immune tolerance (28). Environmental conditions associated with a reduced risk of developing allergic disease include large family size, rural homes, low antibiotic use, and poor sanitation (4, 5, 36, 47). Each of these conditions can be characterized by an increased exposure to microbes and microbial products early in life.

The innate immune system comprises the first line of defense in the lung and initiates an immune response after exposure to microorganisms or foreign antigens. Portions of the innate immune system are present in the developing fetus or newborn, whereas other components are not yet fully functional at birth. The appearance of dendritic cells in the lung occurs during the first year of life (42). Dendritic cells exist in the lung in an immature state. They respond to antigens in the environment by changing their phenotype (35) and can play a pivotal role in influencing the developing immune system (12, 25), specifically lymphocytes. Lymphocytes, in turn, influence the response to microbial or allergic stresses in the environment (17).

Animal models have been devised to explore the influence of endotoxin, a key component of a nonhygienic environment, on the innate immune system and the development of an atopic response. As a model for endotoxin exposure, purified endotoxin is often delivered directly to the airway via intratracheal instillation or as an aerosolized solution. We and others have used murine models in which endotoxin solutions are inhaled to mimic agricultural or domestic exposures (8, 9, 30, 44). Analyses of environmental endotoxin exposure in most epidemiological studies are performed by quantifying endotoxin from airborne dust and dust collected from mattresses and floors (40, 41, 47). When endotoxin levels observed in these domestic and occupational environments are compared with the levels of exposure in animal models, experimental animals are often exposed to considerably higher levels of endotoxin. Importantly, both domestic and agricultural dusts contain endotoxin in addition to other immunologically active components (32). Therefore, studies concerning the influence of complex, naturally occurring organic dusts on the developing lung and immune system are needed to address questions about environmental exposures that cannot be answered by using purified endotoxin.

One aim of the present study was to explore the influence of an endotoxin-containing nonhygienic environment on the susceptibility to atopic airway inflammation. A second goal was to define the pulmonary inflammatory response to this nonhygienic environment during development. We used grain dust, an environmental model of agricultural exposure that is rich in endotoxin and other microbial components, in this study (32). We hypothesized that early life exposure to organic dust with a high concentration of endotoxin would modulate allergic immune responses later in life and also that this exposure

Address for reprint requests and other correspondence: C. L. S. George, Dept. of Pediatrics, 2JCP, Univ. of Iowa, 200 Hawkins Drive, Iowa City, IA 52242 (e-mail: caroline-george@uiowa.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

would be associated with a neutrophilic response in the bronchoalveolar lavage (BAL) fluid. Our experiments demonstrate that subchronic exposures to corn dust either very early in life or later in life attenuate the allergic inflammatory response of the lung in mature animals without modulating systemic levels of ovalbumin-specific IgE or levels of proinflammatory cytokines in BAL fluid. In addition, early life exposure to a corn dust environment did not result in pulmonary neutrophilia but rather increased the numbers of antigen-presenting cells in the lung.

MATERIALS AND METHODS

Materials. All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Environmental exposure. Two types of environmental exposures were created. The hygienic environment (control) consisted of a standard mouse cage lined with Cellu-Dri, a cellulose fiber animal bedding (Shepherd Specialty Papers, Kalamazoo, MI) used by our animal care facility. Dust collected from the grain-drying system located outside at a local grain elevator that processed corn was used as bedding material to create the nonhygienic environment (experimental). Breeding pairs were housed in control bedding or corn dust bedding, and their litters either remained in these environments or were placed into the corn dust bedding as indicated. In preparation for the allergen studies, some mice were born into the control bedding and then were placed into corn dust bedding at 4 wk of age until the end of the study to create a "subchronic" exposure to a nonhygienic environment. Other mice were born and reared into the corn dust bedding and then moved into control bedding at 3 wk of age to create an "early life" exposure to corn dust. Three weeks of age was selected as the end point of the early life exposure to coincide with weaning from the mother.

The control and experimental bedding materials were assessed for endotoxin content by use of the kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay (Whittaker Bioproducts, Walkersville, MD) (38). Our LAL kit conversion from endotoxin units (EU) to nanograms was 9 EU/ng. Ambient dust particles from both environments were collected with a Button aerosol sampler (Eighty Four, SKC), an aerosol-sampling device specifically designed to obtain a representative sample of airborne dust in an environment. The endotoxin content of the airborne particulate matter was assessed by the LAL assay. This allowed us to calculate the concentration of airborne endotoxin (EU/m^3) in cages of individual animals. The size of ambient particles from the control and experimental environments was determined by a Grimm portable dust monitor (series 1.100; Grimm Technologies, Douglasville, GA).

ELISA for (1–6)-branched, (1–3)β-D-glucan. Fungal cell wall components were detected by ELISA, as recently described (22). Briefly, the dust samples were suspended in pyrogen-free PBS with 0.05% Tween 20. Samples were agitated for 1 h at room temperature, autoclaved at 120°C for 1 h, and then centrifuged at 600 *g* for 20 min. The ELISA plates were prepared by coating with a mouse monoclonal anti-(1–6)-branched, (1–3)β-D-glucan antibody (Murex Biotech Limited). A 100-μl sample was applied to the coated plates in serial twofold dilutions. The plates were washed, and then a custom rabbit anti-Scleroglucan polyclonal antibody (1:2,500) was added and incubated for 1 h. The washed plates were incubated with goat anti-rabbit IgG horseradish peroxidase (Bioscience, Camarillo, CA). The washed plates were developed with tetramethylbenzidine substrate, and then the reaction was stopped with 0.18 M H_2SO_4 and read at 405 nm optical density (OD). A standard curve was created with purified Scleroglucan extract (kindly provided by Dr. David William, East Tennessee State University).

Microorganism determination. Microorganisms contained within the corn dust bedding were detected by culture assay by previously described methods (39). Specifically, three types of culture media were employed in duplicate to determine the total bacteria, gram-negative enteric bacteria, and total fungi load within the corn dust bedding.

Animals. Mice (6- to 8-wk-old C3HeB/FeJ) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed together in an isolation cubicle with microisolation lids on each cage. Breeder mice were acclimated to either the control or corn dust environment for 2 wk before they were paired. Litters were maintained in either the corn dust or control bedding environments. Some animals were euthanized on day of life (DOL) 10, DOL 16, or DOL 21 to characterize the effects of the control and corn dust bedding environments on the developing immune system. At 21 days of age, all mice were weaned and placed into a control bedding environment. Weaned mice received water-softened mouse chow (Formulab Chow 5008; Purina Mills, Richmond, IN) until it was determined that they could obtain adequate nutritional support from the dry mouse chow. Mice were provided water and food ad libitum. Animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animal Resources were followed. The University of Iowa Institutional Animal Care and Use Committee approved the protocols used in this study.

Allergen exposure. At 8 wk of age, mice raised in a control bedding environment and mice exposed to corn dust either early in life or as a subchronic exposure starting at 4 wk of age were sensitized and challenged to the allergen ovalbumin as previously described (14, 16). Briefly, the mice were sensitized to ovalbumin via an intraperitoneal injection (100 μl) of 20 μg ovalbumin emulsified in 2 mg alum on allergen exposure *days 0* and *7*. Mice were then challenged by inhalation of ovalbumin (1% solution in normal saline, generated by an Aero-Tech nebulizer, CIS-US) for 30 min on *days 14* and *16*. Animals were then killed on *day 18* of the allergen exposure, at 74 days of age.

BAL cell collection. Mice were euthanized with an overdose of Nembutal (150 mg/kg) and their tracheas were cannulated with PE tubing sized according to their age. We adapted our previously published protocol to accommodate smaller mice (9). The lungs of mice 10–21 days of age were lavaged with 2 ml of pyrogen-free normal saline (Baxter), and adult animals were lavaged with 4 ml of normal saline. Normal saline was allowed to passively flow into and out of the lungs in aliquots ranging from 100 μl to 1 ml according to the size of the animal. The collected lavage fluid was centrifuged at 830 *g* for 5 min at 4°C. The pelleted cells were then resuspended in 200 μl of HBSS. With the use of a hemacytometer and trypan blue staining, a count of living cells was obtained. In our laboratory, 98% of the cells collected from lavage fluid using this technique were live cells. A cytospin preparation of 75–100 μl of the resuspended cells was stained with Hema 3 stain set (Fisher Scientific, Middletown, VA) to obtain a cell type differential count. Cell types counted were macrophages, lymphocytes, neutrophils, and eosinophils. All total and differential cell counts were performed on masked slides by two independent observers and then averaged for each animal.

Cytokine determination. Cytokine identification and quantification from BAL fluid collected from 10-wk-old mice were performed using a multiplex protein kit that utilizes fluorescence antibody capture techniques (Novagen, Madison, WI). The cytokines detected in this assay were IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p70, granulocyte macrophage-colony stimulating factor (GMCSF), IFN-γ, and TNF-α. BAL fluid was collected, and the cellular components were removed by centrifugation as described above. The supernatant was stored at −80°C until analyzed. Once thawed, the samples were diluted 1:1 with assay diluent and assayed per the manufacturer's instructions. The range of cytokine concentration detection was 5–2,500 pg/ml.

Immunoglobulin assay. Our protocol was adapted from a previously described method (29). Briefly, serum levels of ovalbumin-specific IgE were measured by capture ELISA. Ninety-six-well plates

were incubated with 10 mg/ml ovalbumin in RPMI 1640 medium. After an overnight incubation, wash, and blocking step with 10% FCS in PBS, duplicate samples of serum (1:50 dilution) were incubated overnight in the plate at 4°C. After a washing step, ovalbumin-captured IgE was detected with biotinylated rat anti-mouse-IgE (clone R35–118, BD PharMingen, San Diego, CA). After another washing step, avidin peroxidase (BD PharMingen) was added to each well. The reaction was developed by adding tetramethylbenzidine substrate (BD PharMingen) after a final washing step. Units were reported as OD readings at 450 nm. The control, unexposed animal values were made equal to 1, and the values for the exposed animal were adjusted accordingly.

Immunohistochemistry. Mice (21 days old) were euthanized, their trachea cannulated with PE-50 tubing, and their lungs inflated with 0.5 ml 70% OCT compound (Sakura Finetek USA, Torrance, CA) in PBS and then frozen in an acetone-dry ice bath. Frozen sections of lung tissue (5 μm) were first fixed in cold acetone-ethanol (3:1), pretreated with 0.3% hydrogen peroxide in PBS containing 0.3% horse serum, and then blocked with an avidin and biotin blocking kit (Vector Laboratories, Burlingame, CA). The tissue sections were then incubated with an MHC class II anti-mouse I-E^k antibody (clone 17-3-3, BD PharMingen). The "Mouse on Mouse" light microscopy or fluorescein kits from Vector were used to process the tissue sections per instructions from the manufacturer. Slides for light microscopy were developed with 4% diaminobenzedine and counterstained with Meyer's hematoxylin. Light microscopy images of lung tissue were captured with a Spot Jr. digital camera. Dendritic cells were similarly identified with an antibody specific for the surface marker CD11c (clone N418, Serotec, Raleigh, NC) and processed with streptavidin Alexa Fluor 568 for immunofluorescent staining. The antibody diluent, PBS, was used without the primary antibody as a control. Confocal microscopy images of immunofluorescence were captured with a Zeiss 510 microscope. For quantitative analysis of the antigen-presenting cells within lung tissue, two individuals blinded to the condition counted the number of I-E^k-positive cells per ×20 alveolar field or the number of positive cells within the intraepithelial area of a conducting airway using light microscopy. Consecutive alveolar images from the inferior portion of the left lung were obtained avoiding large blood vessels and conducting airways. As previously described, images of conducting airways were collected, and obliquely cut conducting airways were not analyzed (8). To obtain the ratio of CD11c+ I-E^k+ cells to CD11c− I-E^k+ cells, two observers counted 100 consecutive fluorescent cells. The data were collected from four or five images/animal and averaged between the two individuals.

Assessment of perivascular inflammation. Adult mice were euthanized, their right heart flushed with PBS, and their trachea cannulated with PE-90 tubing. The lungs were inflated with zinc-formalin solution (LABSCO, Louisville, KY) at a pressure head of 25 cmH_2O. Whole lungs were processed into paraffin blocks and sectioned posterior to anterior in a caudal-cranial plane. Lung tissue sections 5 μm thick, obtained 75 μm from the posterior aspect, were mounted onto glass slides. These lung sections were stained with hematoxylin and eosin. A perivascular inflammatory grading scale was adapted from a previously published study that focused on the number and density of mononuclear cells within the perivascular areas of interstitial blood vessels (18). Our scale ranged from 1 to 4, with grade 1 defined as none to few mononuclear cells (as typically seen in the majority of control animals), grade 2 defined as mononuclear cells 1 or 2 cells thick around the vessel, grade 3 defined as perivascular areas two to three cells thick surrounding the vessel, and grade 4 defined as densely packed (>5 cells thick) inflammatory cells of at least ~50% of the vessel. Two individuals blinded to the condition assessed 100 blood vessels per animal and graded them according to the degree of mononuclear inflammatory cells in the perivascular area. The percent of blood vessels in each grade for each animal (n = 7–10 animals/group) was averaged between the two observers.

Statistics. Student's two-sample t-test was used to compare means between two groups for normally distributed data. For comparing multiple groups to test for the effect of two factors (i.e., exposure condition and age) and their interaction, 2-way ANOVA was used. Bonferroni adjustment of the P value was applied when multiple comparisons were performed. For variables that were not normally distributed, such as cell count, a geometric mean was calculated. A P value of <0.05, or less than the critical P value using the Bonferroni adjustment, was considered statistically significant. All data are expressed as means ± SD. The degree of interobserver agreement of the inflammatory score was measured by the interobserver coefficient of variation and the intraclass correlation, with an intraclass correlation >0.75 considered to be very good agreement. The interclass correlation between the two observers was 82% with 95% confidence intervals. All the statistical analysis for this study was performed with SigmaStat 3.0 software.

RESULTS

Characterization of the environmental exposures. The corn dust that was used as bedding material contained a 57 times greater concentration of endotoxin compared with the control bedding [1,246 ± 341 EU/mg in the corn dust (0.14 μg/mg) vs. 22 ± 13 EU/mg (0.002 μg/mg) in the control bedding; $P <$ 0.001]. Endotoxin measurements were also obtained from control bedding animal cages with three mice per cage, at four time points (*time 0*, 24 h, 72 h, and 7 days). The mean endotoxin concentration in the control bedding over time was 5.55 ± 6.42 EU/mg bedding, with a range of 1.78–15.12 EU/mg. Therefore, the contribution of mice living in a cage over time toward the total endotoxin exposure was negligible compared with the high concentration of endotoxin in the corn dust. The corn dust bedding contained 0.27 μg/mg (1–3)β-D-glucan, 760 colony forming units (CFU)/mg culturable fungi, 540 CFU/mg culturable mesophilic bacteria, and 30 CFU/mg culturable thermophilic bacteria. In comparison, the control bedding contained <10 CFU/1,000 mg fungi and <3 CFU/1,000 mg mesophilic bacteria. Size distribution analysis of ambient particles obtained from representative animal cages that contained either corn dust or control bedding demonstrated that over 90% of airborne particles were smaller than 1 μm aerodynamic diameter in both environments (Fig. 1). The average concentration of airborne particulate matter in the corn dust bedding environments was similar to the control bedding environments (0.61 ± 0.59 mg/m^3 vs. 0.49 ± 0.41 mg/m^3, respectively). The inhalable airborne endotoxin concentration averaged 620 ± 198 EU/m^3 (0.07 μg/m^3) in the corn dust bedding environments and was significantly higher ($P <$ 0.001) than in the control environments [43 ± 1.78 EU/m^3 (0.005 μg/m^3)].

Both early life and later life subchronic exposure to corn dust reduce atopic airway inflammation. The airway response to sensitization and challenge with ovalbumin in mice raised in control bedding was compared with that of mice who experienced a subchronic corn dust bedding exposure (4 wk of life through the end of the experiment) or an early life exposure to corn dust (from conception through 3 wk of age). Ovalbumin-treated mice raised in control bedding developed significant BAL pleocytosis and eosinophilia compared with untreated mice (Fig. 2*A*; $P < 0.001$). Mice with a subchronic exposure to the corn dust bedding developed significantly less cellular airway inflammation (Fig. 2*A*; $P < 0.01$) and BAL eosinophilia (Fig. 2*B*; $P < 0.05$) than did those mice raised in control

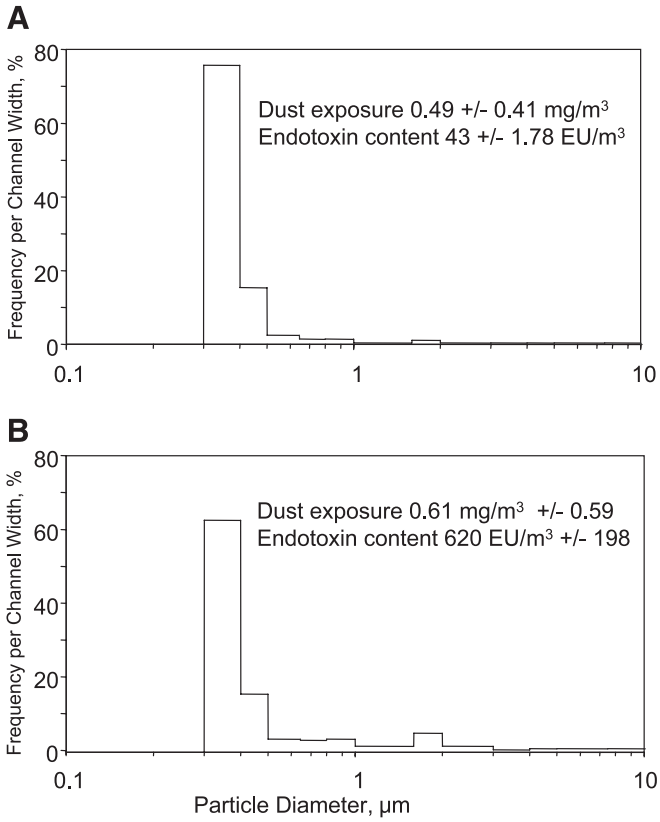


Fig. 1. Graphic representation of particle size data collected from 2 mouse cages containing control (*A*) or corn dust (*B*) bedding. Data represent percentage of ambient particles counted in each size channel by a Grimm portable dust monitor. Ambient particle endotoxin concentrations were assayed during five 24-h sample collection periods throughout the experimental period using a Button sampler, gravimetric analysis, and the *Limulus* amebocyte lysate assay. The dust exposure and endotoxin data are presented as means ± SD. EU, endotoxin unit.

bedding. Interestingly, mice with only an early life exposure to corn dust bedding also demonstrated significantly fewer total BAL cells (Fig. 2*A*; $P < 0.01$) as well as BAL eosinophilia (Fig. 2*B*; $P < 0.05$) compared with mice reared in control bedding, in response to ovalbumin treatment.

Early life exposure to corn dust bedding alters the perivascular but not peribronchial inflammatory response. Histological examination of lung tissue demonstrated peribronchial inflammatory changes after ovalbumin treatment compared with mice not treated with ovalbumin (Fig. 3). Similar inflammatory changes around the conducting airways were observed in ovalbumin-treated mice reared in either control bedding or corn dust bedding. However, a key difference between the groups was seen in ovalbumin-induced development of a perivascular monocytic infiltrates (Fig. 4*A*). Using an inflammation scoring method for perivascular responses (18) (Fig. 4*B*), we found that mice with early life exposure to corn dust demonstrated a significant reduction in intensity of perivascular inflammation compared with control mice, after ovalbumin-treatment (Fig. 4*C*) (18). There was a significant decrease ($P < 0.05$) in ovalbumin-induced grade 3/4 perivascular inflammation in mice exposed to corn dust early in life and a corresponding increase in grade 1/2 perivascular inflammation (Fig. 4*C*).

Corn dust exposure does not alter the induction of ovalbumin-induced antibodies. Despite a modulation of ovalbumin-induced airway inflammation by early life exposure to corn dust, the systemic response to ovalbumin was not affected. This was demonstrated by an ELISA determination of ovalbumin-IgE levels in serum that showed there was no difference between ovalbumin-treated mice with and without an early life exposure to corn dust (OD = 15.08 ± 2.74 and 18.40 ± 2.20, respectively; $P > 0.1$, determined by Student's *t*-test, from 3 separate experiments).

Corn dust bedding exposure does not induce pulmonary inflammation. To evaluate whether exposure to the corn dust alone may induce significant airway inflammation, we evaluated the pulmonary cellular response of adult mice from the four environmental exposure groups. We found no significant difference in total BAL fluid cell number (Fig. 5*A*) or airway neutrophilia (Fig. 5*B*) between the control mice or mice exposed to corn dust early in life, later in life, or continuously. The majority of BAL cells in all animals were alveolar macrophages (>85%), whereas neutrophils averaged only 7% of

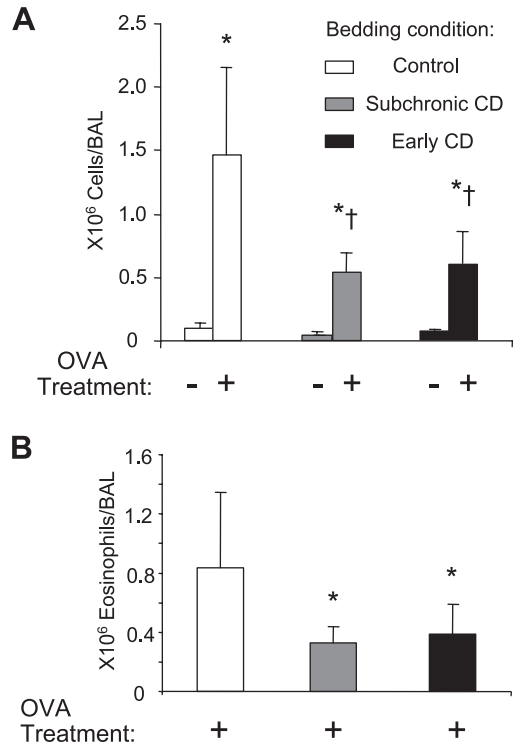


Fig. 2. *A*: number of total cells in bronchoalveolar lavage (BAL) fluid in 74-day-old mice, with (+) and without (−) ovalbumin (OVA) treatment. Animals treated with OVA had significantly more cells compared with non-OVA-treated animals (*$P < 0.001$ by 2-way ANOVA). OVA-treated mice with a subchronic exposure to corn dust (CD) bedding (from 4 wk of life until the end of the OVA treatment) had significantly fewer cells in BAL fluid than untreated control mice (control + OVA vs. subchronic CD + OVA; †$P < 0.01$, 2-way ANOVA). Ova-treated mice with an early life exposure to corn dust bedding (from conception until 3 wk of life) also had a significant decrease in total cells in BAL fluid compared with OVA-treated control bedding mice (control + OVA vs. early CD + OVA; †$P < 0.01$). *B*: eosinophils in BAL fluid were significantly reduced in number after OVA treatment in mice with either a subchronic or early life exposure to corn dust (*$P < 0.05$, Student's *t*-test) compared with mice reared in control bedding. Mice that did not receive OVA treatment do not have eosinophils in their BAL fluid (data not shown). Data are means ± SD; $n = 10–17$ animals/condition.

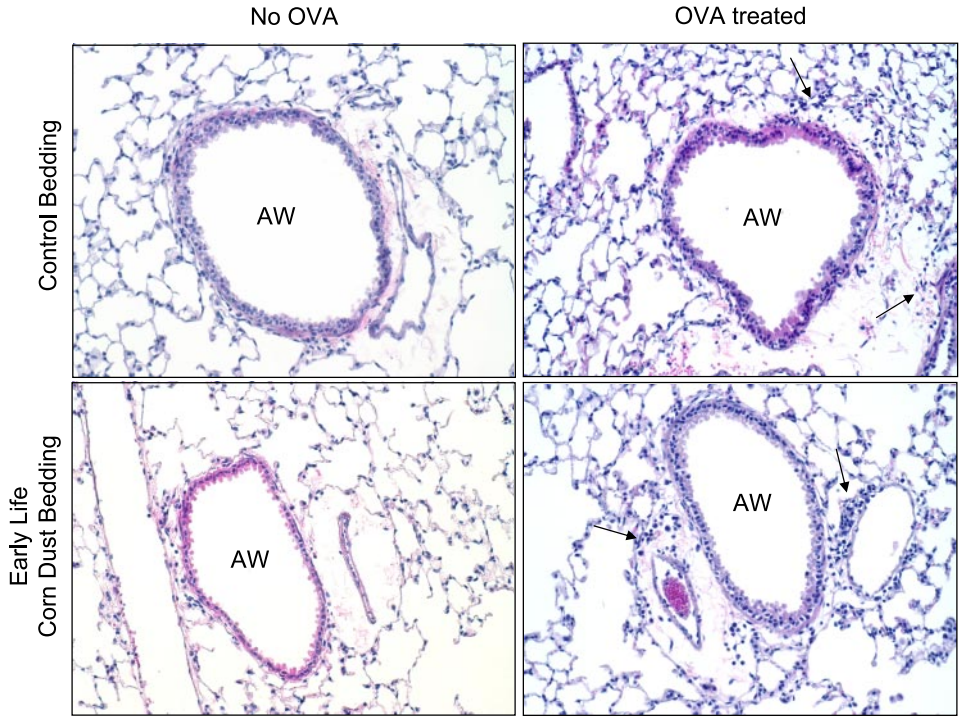


Fig. 3. Representative photomicrographs of hematoxylin and eosin-stained lung tissues from mice with and without OVA treatment, with and without early life exposure to corn dust bedding. OVA-treated mice have mononuclear-infiltrating cells present near the conducting airways (AW) in both early life environmental conditions (arrows), whereas mice not treated with OVA lack these infiltrating cells. No difference in the OVA-induced inflammatory response surrounding the conducting airways was observed when comparing the 2 early life exposure groups.

the BAL cells. Cytokine analysis of BAL fluid similarly demonstrated that exposure to corn dust-bedding did not significantly alter the concentration of IL-6, IL-10, IL-12, GMCSF, IFN-γ, or TNF-α, compared with control bedding exposed animals (Fig. 6). Interestingly, the number of neutrophils and the concentration of TNF-α in BAL fluid were lowest in the subchronic group; however, these changes were not statistically significant. Other cytokine proteins detected in this assay were below the level of detection in the BAL fluid.

Maturation of pulmonary immune cells. To better understand the immune effects of early life environment, control and corn dust-exposed mice were compared at several time points. Between DOL 10 and DOL 21, there was a rapid and significant increase in the number of immune cells present in BAL fluid (Fig. 7*A*). At DOL 10, eosinophils comprised a sizable proportion of the BAL cells isolated from mice raised in control bedding as well as those raised in corn dust bedding (Fig. 7*B*), consistent with the understanding of the normal early life immune environment being Th2 skewed. During this period of development, mice reared in corn dust bedding demonstrated significantly more eosinophils compared with control mice ($P < 0.05$). As the animals mature, the eosinophil numbers decreased toward adult levels (~0.5% of total BAL cells), whereas alveolar macrophages increased proportionately throughout maturation (data not shown). Mice reared in the corn dust bedding tended to have increased numbers of neutrophils in their BAL fluid; however, this difference did not reach statistical significance ($P = 0.054$, DOL 10–21) (Fig. 7*C*). By DOL 21, mice reared in either environmental condition had a BAL fluid cell profile similar to that observed in adult mice (9).

Pulmonary antigen-presenting cells in the lung parenchyma were evaluated by immunostaining. MHC class II ($I\text{-}E^k$)-positive cells were detected within the epithelium of the conducting airways and surrounding areas as well as within alveolar walls (Fig. 8*A*). At DOL 21, there was a significant ($P < 0.01$) increase in the number of MHC class II-positive cells within the alveolar spaces of mice reared in corn dust compared with the number of cells in mice reared in control bedding (Fig. 8*B*). There was, however, no significant difference in the number of MHC class II-positive cells within the epithelium of conducting airways in mice reared in control bedding vs. those reared in corn dust (Fig. 8*B*).

We hypothesized that the increased antigen-presenting cell populations in the lung were likely to be dendritic cells. This was suggested by the localization of the MHC class II-positive cells within the lung tissue walls and colocalization of $I\text{-}E^k$ (MHC class II protein) and CD11c (common dendritic cell surface marker) in all of the cells within the conducting airway epithelium and the majority of cells within the alveolar wall (Fig. 9*A*). The proportion of CD11c+ $I\text{-}E^k$+ cells (dendritic cells) to CD11c− $I\text{-}E^k$+ cells (macrophages) was similar between mice raised in control and corn dust conditions (Fig. 9*B*).

DISCUSSION

Many have theorized that environmental exposures can tip the developing immune system toward either the expression of genes associated with allergic disorders or toward nonallergic genes (48). Our murine model of environmental exposure to a "nonhygienic" dry organic dust environment has allowed us to investigate the influence of early life environment on the developing pulmonary immune system. In this study, we first demonstrated that the corn dust environment contains respirable particulate matter. In mice, and other small rodents, particles <1 μm aerodynamic diameter are likely to reach the alveolar region (13). The composition of our corn dust bedding was complex. We confirmed that the level of ambient endotoxin in the corn dust environment was significantly higher than in the control bedding environment. Additionally, the corn

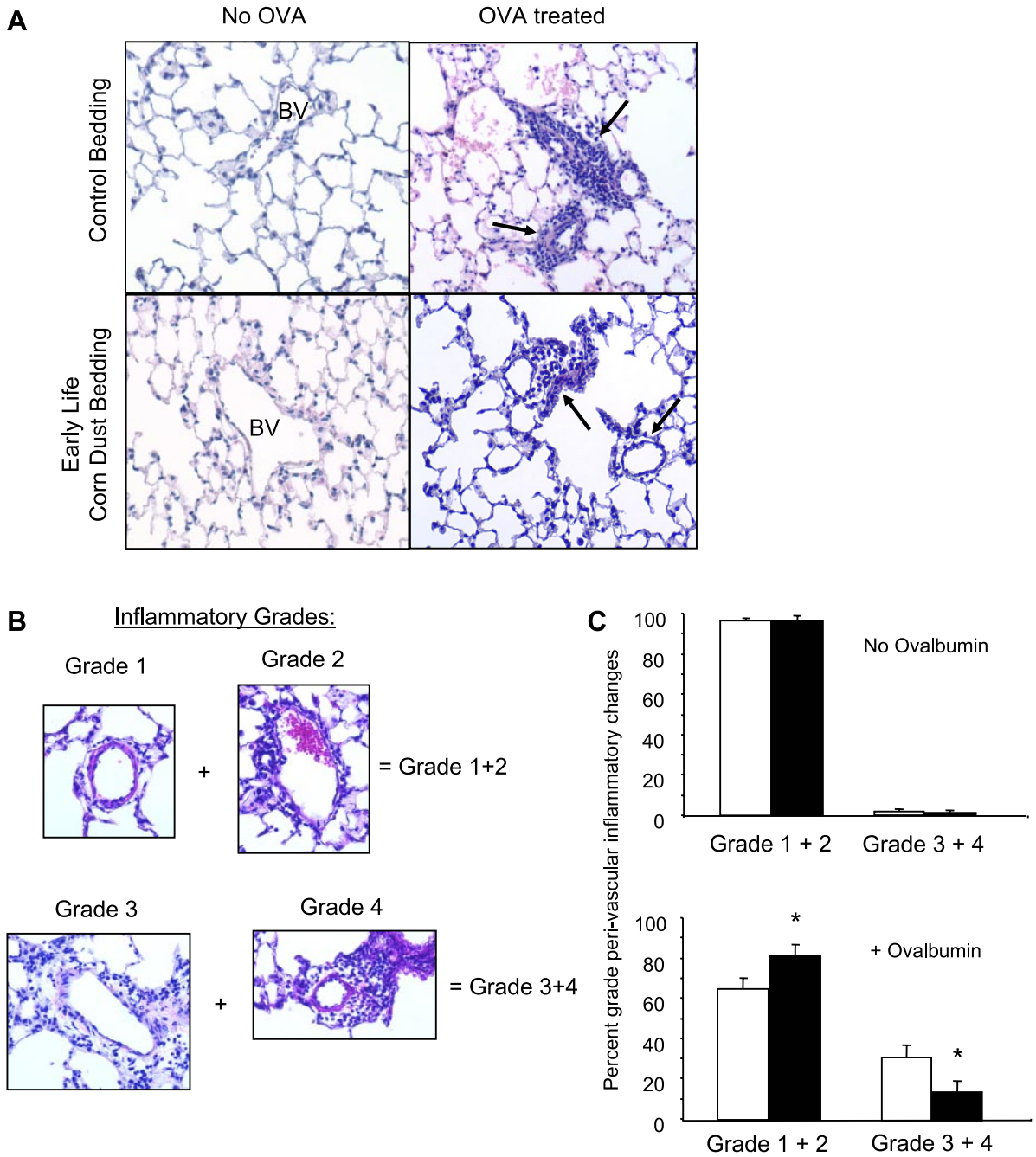


Fig. 4. *A*: representative photomicrographs of blood vessels (BV) within lung tissue with and without OVA treatment. Mice that were not treated with OVA did not demonstrate perivascular inflammation. Typical inflammatory changes after OVA treatment in the perivascular regions are indicated (arrows). Less perivascular inflammation was observed in the lungs of mice exposed to corn dust bedding early in life compared with those mice that were reared in control bedding. Although both early life exposure groups had OVA-induced mononuclear inflammatory cell infiltrates around blood vessels, mice reared in corn dust bedding had less interstitial inflammation than mice reared in control bedding. *B*: grading scale used to assess perivascular inflammation. Grades 1–4 represent a progressively increasing density of mononuclear inflammatory cells in a circumferential pattern around blood vessels. *C*: percentage of blood vessels within each grade of inflammation; 100 blood vessels were graded from each animal. Early life exposure to corn dust (black bars) significantly decreased ($*P < 0.05$) the number of lung blood vessels with grade 3/4 inflammation and increased the number of blood vessels with grade 1/2 inflammation after OVA treatment compared with mice reared in control bedding (white bars). Data are means ± SD; $n = 7$–10 animals/condition.

dust bedding also contained microorganisms and immunologically active branched β-glucans of fungal origin. Like endotoxin, β-glucans can favor production of a Th1 cytokine profile (37). When administered orally in a swine model of influenza infection, β-glucans both increase IFN-γ in the lung and decrease the viral load (15). In summary, our murine model contains several components in addition to endotoxin that are immunologically active.

We chose to focus on the inflammatory response to an allergen as an endpoint because both animal models and epidemiologic studies of large populations have found a close association between environmental exposure to endotoxin and

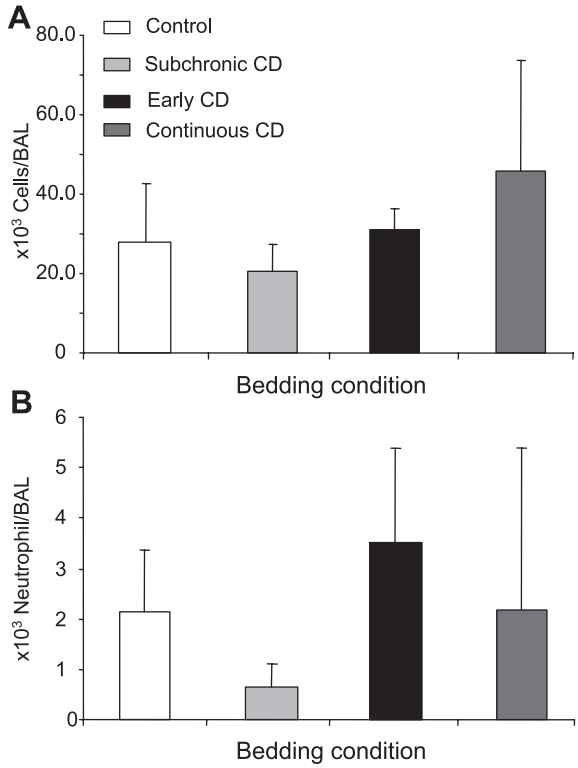


Fig. 5. *A*: numbers of cells in BAL fluid. Cell counts were determined in 74-day-old mice that experienced 1 of 4 environmental conditions (control bedding, and subchronic, early life, or continuous exposure to corn dust bedding). There were no significant differences in cell numbers between any of the corn dust bedding exposures and the control mice. *B*: number of neutrophils in BAL fluid. There were no significant differences in the total number of neutrophils in any of the corn dust exposures compared with control mice. Data are means ± SD; $n = 4$ animals/condition.

markers of atopy (5, 10, 11, 21). Mice who experienced a subchronic exposure to corn dust bedding (from 4 to 10.5 wk of life) were characterized by an attenuated lung cellular inflammatory response as reflected by the number of total BAL fluid cells and eosinophils. A similar reduction in eosinophilia

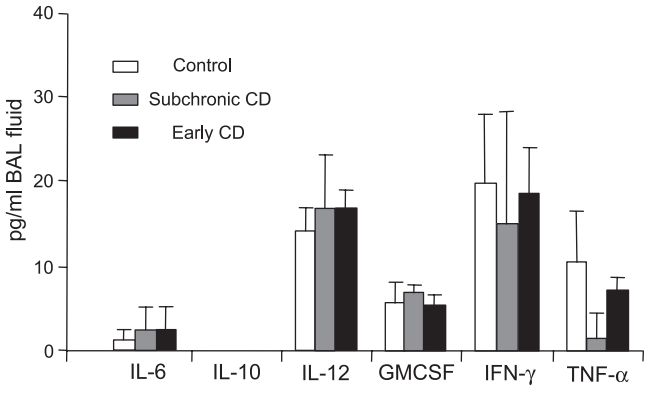


Fig. 6. Cytokine levels in BAL fluid. Cytokines were measured in BAL fluid obtained from 74-day-old mice that had been exposed to either control bedding or subchronic corn dust bedding (from 4 wk to 74 days of life) or to early life corn dust bedding (from conception until 3 wk of life). There were no significant differences between the control mice and the 2 corn dust bedding exposure groups in the levels of IL-6, IL-10, IL-12, granulocyte macrophage-colony stimulating factor (GMCSF), IFN-γ, or TNF-α. Data are means ± SD; $n = 3$ or 4 animals/condition.

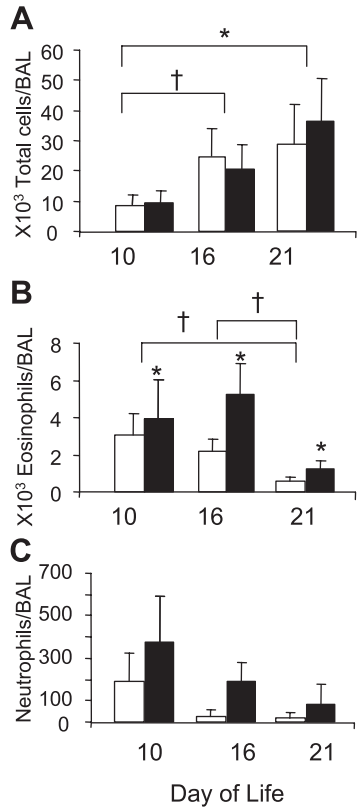


Fig. 7. BAL white blood cell (WBC) populations during development. *A*: total WBC counts in mice reared in control bedding (white bars) vs. in corn dust bedding (black bars) were determined at day of life (DOL) 10, DOL 16, and DOL 21. The total number of BAL cells increased with increasing age (*$P <$ 0.05, DOL 10 vs. DOL 21; †$P < 0.01$, DOL 10 vs. DOL 16; 2-way ANOVA). There was no difference between the number of BAL cells in mice reared in the control and corn dust environments at any stage of development. *B*: eosinophil numbers in BAL fluid. The total number of eosinophils obtained from BAL fluid was significantly greater in the corn dust-reared mice compared with the control mice (*$P < 0.05$, 2-way ANOVA). Over time, the eosinophil number decreased with age in all mice. *C*: neutrophil numbers in BAL fluid. Mice reared in corn dust bedding tended to have increased numbers of BAL neutrophils compared with the control-bedding mice; however, the difference was not statistically significant ($P = 0.054$, 2-way ANOVA). The number of neutrophils in both environments decreased with age. Data are means ± SD; $n = 7$–13 animals/condition at each age.

has been demonstrated by Tulic et al. (43) in a rat model of ovalbumin-induced lung injury when the animals were treated with intraperitoneal LPS before or just after the intraperitoneal sensitization dose of ovalbumin (43). In our studies, we also bred mice into the corn dust environment and then removed them at 3 wk of age to limit their environmental exposure. With this experimental design, our results show that exposure to the corn dust environment early in life also can attenuate the inflammatory response that the adult mouse develops in response to an allergen. A similar reduction in ovalbumin-induced BAL pleocytosis and eosinophilia has been described recently in a model of in utero and perinatal exposure to LPS (3).

In addition to a reduction in BAL pleocytosis and in eosinophil numbers, the mice exposed to the corn dust bedding environment early in life also had reduced pulmonary interstitial inflammation. Typically, murine models of ovalbumin-induced allergic lung injury are characterized by peribronchial and perivascular inflammation. Whereas all animals treated

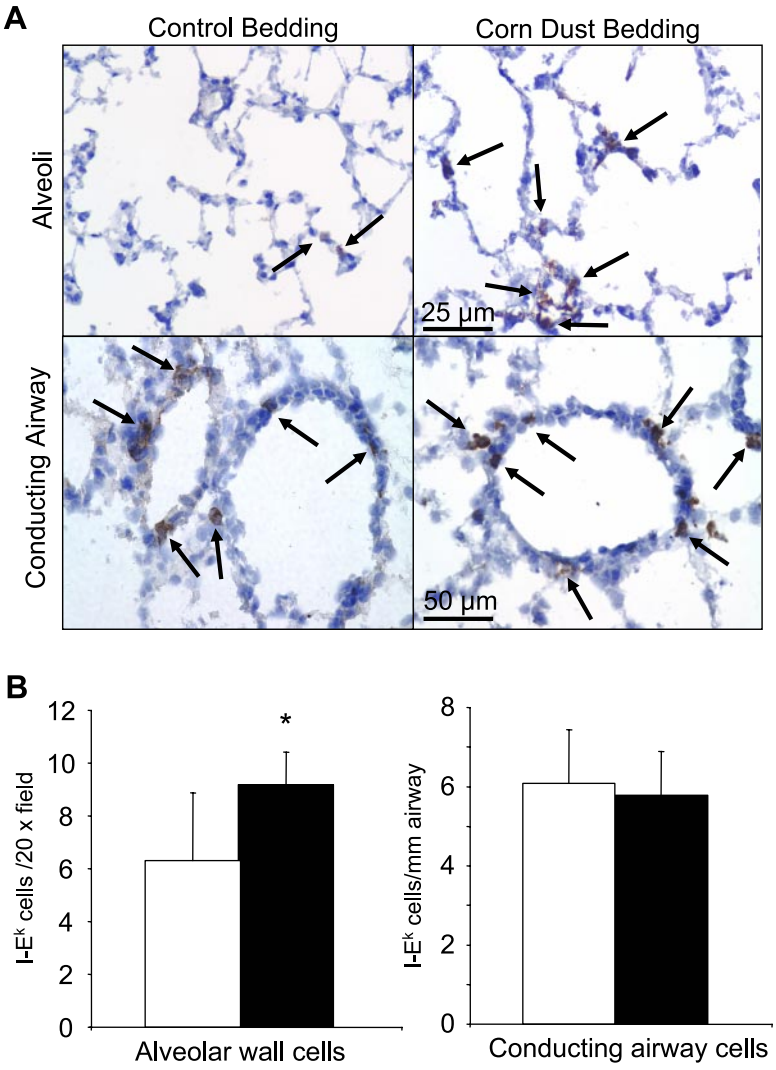


Fig. 8. Major histocompatibility complex (MHC) class II cells in the lung. Representative photomicrographs from 21-day-old mice reared in control and corn dust bedding. *A*: cells that stain positive for the I-E^k protein (MHC class II positive cells) are brown and are indicated with arrows. *B*: quantitation of MHC class II cells in the lung. Corn dust bedding (black bars) did significantly increase the number of MHC class II + antigen-presenting cells in alveolar wall tissue but did not affect the number of antigen-presenting cells in conducting airways of 21-day-old mice, compared with cell numbers in mice reared in control bedding (white bars). Data are means ± SD; $n = 4$ or 5 animals/condition. *$P < 0.01$.

with ovalbumin in the present study were observed to have mononuclear inflammation in the peribronchial region, one obvious difference in the lung histology of mice exposed to corn dust early in life was the reduced degree of mononuclear infiltrating cells surrounding blood vessels. Most studies that used murine models of allergen-induced lung inflammation do not distinguish between various compartments of lung when evaluating interstitial inflammation or limit the focus to the peribronchial compartment. The assumption that the respiratory tract beyond the level of the mainstem bronchi is uniform in immune system function may not be correct. Legge and Braciale (19) demonstrated that the dendritic cell population associated with pulmonary conducting airways have a rate of turnover different from those in the alveolar compartment. Moreover, dendritic cell activity in the conducting airways and peripheral lung tissue may be under the control of local mediators that create unique microenvironments in these sites (46). Finally, in a murine model of inhalation exposure, it was shown that diesel exhaust particles induce perivascular inflammation that develops in the absence of peribronchial inflammation. This is in contrast to ovalbumin-induced inflammatory changes where both lung compartments (perivascular and peribronchial) developed mononuclear infiltrates (34). Therefore, it is possible that the perivascular and peribronchial immune system compartments in the lung are differentially affected by stimuli and this accounts for the altered perivascular histology that we observed after ovalbumin treatment.

In contrast to the pulmonary response to ovalbumin, serum ovalbumin-IgE levels were unaffected by the corn dust environment. Others have demonstrated that when the endotoxin is delivered systemically, even in the prenatal period, serum ovalbumin-IgE levels are decreased (3). One significant difference between our experimental design and other models is that our endotoxin exposure utilizes spontaneous inhalation of respirable microbial products, whereas others use intraperitoneal injections of LPS (3) or aerosolization of purified endotoxin (44). Our results, in contrast to these other models of endotoxin exposure, do not support the epidemiological studies that found an association with endotoxin-laden dust in agricultural domestic settings and a reduced incidence of atopy (21, 47). One reason that our exposure model did not attenuate the systemic allergic response may be that the corn dust bedding environment is a less potent stimulus compared with the delivery of purified endotoxin, as suggested by the different immune responses associated with high- vs. low-dose endotoxin exposures (6).

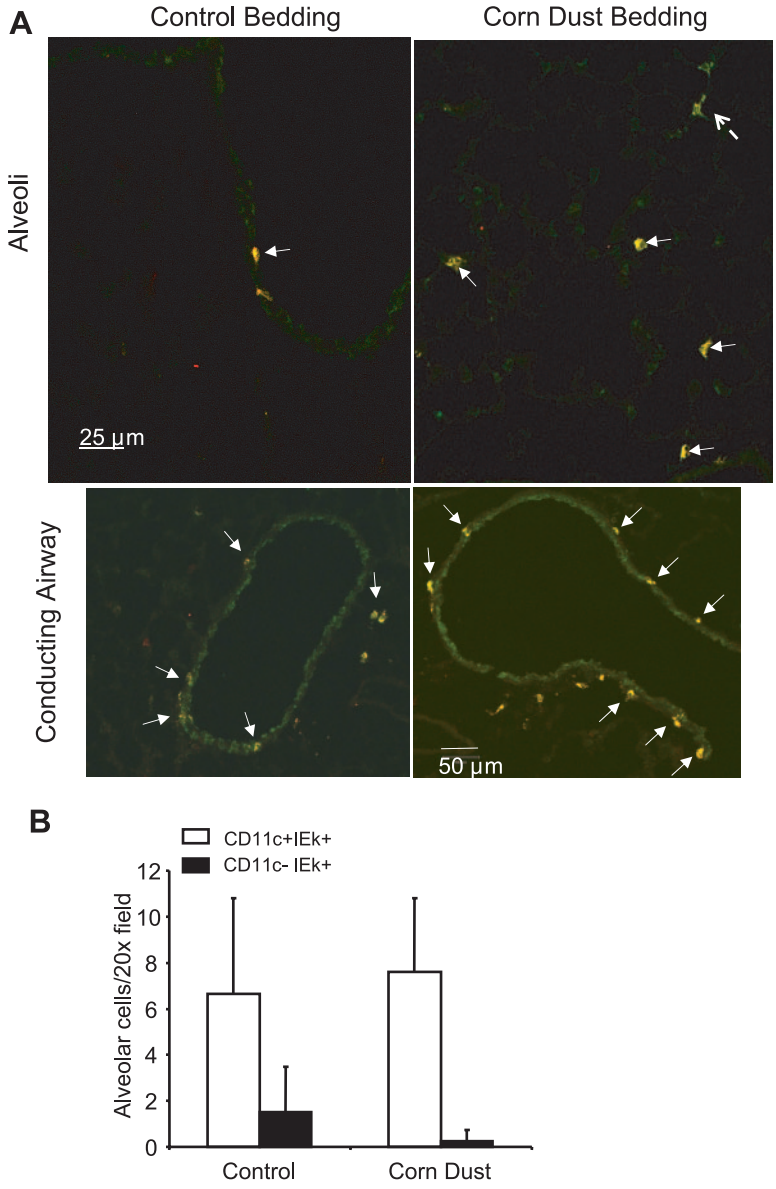


Fig. 9. Antigen-presenting cells in the lung. *A*: representative images of immunofluorescence staining from 21-day-old mice demonstrate that MHC class II-positive cells (I-E^k antibody, green color) also stain for CD11c (a dendritic cell marker, red color). Colocalization of both antigens is indicated by yellow fluorescence (solid arrows) and was detected in both the conducting airways and in the alveolar walls. A minority of cells in the alveoli stained for the MHC class II protein I-E^k only (dashed arrows), and these may represent macrophages. *B*: ratio of CD11c-positive MHC class II-positive cells to CD11c-negative MHC class II-positive cells. There was no significant difference in the ratio of the CD11C+ I-E^k+ to CD11c− I-E^k+ cells present in the control or corn dust-exposed mice. CD11c+ I-E^k− cells made up <1% of the total cell population in both groups of mice. Data are means ± SD; n = 3 animals/condition.

The corn dust bedding environment was not associated with an inflammatory response in pulmonary alveoli at any time point examined in this study. We specifically chose the C3HeB/FeJ strain of mouse for our studies because it is particularly sensitive to endotoxin with respect to the recruitment of neutrophils into the lower airways (20). We anticipated that an environment with endotoxin levels comparable to previously described agricultural and domestic settings (10, 47) would be associated with neutrophilia. The lack of a significant increase in the number of neutrophils in BAL fluid both in the very young or mature mouse makes our corn dust endotoxin exposure very different from previously published aerosolized endotoxin models where a significant influx of neutrophils into the lung is observed (8, 44). An increase in the number of neutrophils tends to reflect the endotoxin level delivered to the lung. The endotoxin concentrations in the respirable portion of the airborne corn dust environment was equal to or higher than what has been observed in domestic settings (47). However, it is difficult to compare our dust exposure to animal models presented in the literature, as many deliver a set amount of purified endotoxin directly to the airway (6), whereas others do not report the airborne concentration when a nebulizer apparatus is utilized (44). Furthermore, it is possible that animal models that use high-dose purified endotoxin exposures to study endotoxin-induced immunomodulation may produce a very different immune system response that might be occurring in the rural household exposures described in the hygiene hypothesis. Finally, because our mice did not demonstrate the expected neutrophil response to endotoxin, yet demonstrated an attenuated response to the ovalbumin, other immunologically active components within a nonhygienic environment may be important in altering allergic immune responses.

We failed to identify alterations in the BAL fluid of either proinflammatory cytokines (TNF-α and IL-6) or in moderating cytokines (IL-10, IL-12, IFN-γ, and GMCSF) compared with levels in control mice. These results were unexpected because we and others have demonstrated that repeated exposure to

aerosolized or intratracheal delivery of endotoxin-containing solutions is associated with the increased production of several proinflammatory cytokines (8, 45). Because the corn dust bedding environment did not alter BAL fluid cytokine levels or neutrophil numbers, we speculated that we might find alterations within the immune cells earlier in life and that with time the animal had developed a tolerance to this environment.

We next focused on the immune-modulating effects of corn dust on immature mice. We report here for the first time that normal inbred mice demonstrate significant airway eosinophilia early in life. This predominance of eosinophils decreases rapidly during postnatal lung development. The high concentration of lung eosinophils early in life may be associated with the Th2 bias (or reduced Th1 presence) previously described in neonatal mice (1, 2, 33) and human infants (49). The presence of relatively large numbers of eosinophils in the alveolar space early in development has not previously been reported in human or murine studies and may be specific to this strain of mouse. This finding may be important because the change in eosinophil numbers occurs during a critical time during immune system development (7) and may influence how this strain of mouse responds to allergen stimulation. Both the control and corn dust environmental exposures were associated with increased eosinophil numbers during the first 3 wk of life compared with numbers in adult mice. In newborns and infants during the first year of life, failing to shift a Th2 cytokine profile to a Th1 profile has been associated with blood eosinophilia and serum markers of atopy (24). Others have demonstrated that there is no relationship between the number of eosinophils and an attenuated late asthmatic response with repeated low-dose allergen exposure (26). Therefore, investigation into the Th1/Th2 balance in very young mice outside of the BAL fluid compartment may help clarify the association between early life eosinophilia and a reduced later life allergic inflammatory response.

On completion of the early life exposure to corn dust at DOL 21, we detected increased numbers of antigen-presenting (dendritic) cells in lung tissue compared with the numbers in mice of the same age reared in control bedding. This increase was observed in alveolar walls but not in the conducting airways. This may reflect a more rapid turnover of dendritic cells in the upper airways compared with lower respiratory tract dendritic cells (19). It is unclear where the corn dust particles impact the lung and come into contact with the immune system in our murine model. However, given the delicate nature of the alveolar spaces required for gas exchange, it was anticipated that components of both the innate and phagocytic immune systems may be increased in animals exposed to the experimental nonhygienic environment.

The exposure to corn dust in our experimental model was complex and unique, involving not only the respiratory system but also the gastrointestinal system because it is likely that dust particles were ingested during grooming. A state of immune system tolerance can be induced after either ingestion or inhalation of immunoreactive substances. Specifically, in a murine model, oral administration of ovalbumin before intraperitoneal sensitization prevents the development of allergen-induced lung inflammation (31). Similarly, exposure of the nasal mucosa to ovalbumin before intraperitoneal sensitization also attenuates the lung's atopic inflammatory response (23). Therefore, it is possible the gastrointestinal tract, in addition to the upper and lower respiratory tracts, contributed to the immune system changes that we observed in this study.

The original description of the hygiene hypothesis proposed that smaller family size, reduced infections early in life, and exposure to a more hygienic environment were associated with an increased prevalence of atopic disease (36). More recent studies support the idea of a critical period early in development in which environmental or infectious exposures can render the individual prone to either atopy or tolerance (27). Our results support this idea by demonstrating that early life exposure to a nonhygienic environment can influence how the mature individual responds to an allergic stimulus. We have also demonstrated that the corn dust exposure does not cause significant alterations in the cellular and cytokine protein composition of BAL fluid but results in more subtle alterations, specifically in the population of interstitial antigen-presenting cells. This novel animal model will allow investigation into the impact of diverse and complex environmental exposures on the developing immune system in the respiratory tract.

ACKNOWLEDGMENTS

The authors acknowledge Marsha O'Neill and Dr. Nervana Metwali for assistance with microbial analysis, glucan, and endotoxin measurements.

GRANTS

This work was funded by the American Lung Association; National Institutes of Health Grants ES-05605, HL-59324, HL-079448, and HL-50050; and a Parker B. Francis Pulmonary Fellowship.

REFERENCES

1. **Adkins B.** T-cell function in newborn mice and humans. *Immunol Today* 20: 330–335, 1999.
2. **Barrios C, Brandt C, Berney M, Lambert PH, and Siegrist CA.** Partial correction of the TH2/TH1 imbalance in neonatal murine responses to vaccine antigens through selective adjuvant effects. *Eur J Immunol* 26: 2666–2670, 1996.
3. **Blumer N, Herz U, Wegmann M, and Renz H.** Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma. *Clin Exp Allergy* 35: 397–402, 2005.
4. **Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, and von Mutius E.** Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 347: 869–877, 2002.
5. **Chrischilles E, Ahrens R, Kuehl A, Kelly K, Thorne P, Burmeister L, and Merchant J.** Asthma prevalence and morbidity among rural Iowa schoolchildren. *J Allergy Clin Immunol* 113: 66–71, 2004.
6. **Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, and Bottomly K.** Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196: 1645–1651, 2002.
7. **Forsthuber T, Yip HC, and Lehmann PV.** Induction of TH1 and TH2 immunity in neonatal mice. *Science* 271: 1728–1730, 1996.
8. **George C, Jin H, Wohlford-Lenane C, O'Neill M, Phipps J, O'Shaughnessy P, Kline J, Thorne P, and Schwartz DA.** Endotoxin responsiveness and subchronic grain dust induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 280: L203–L213, 2001.
9. **George CL, White ML, O'Neill ME, Thorne PS, Schwartz DA, and Snyder JM.** Altered surfactant protein-A gene expression and protein metabolism associated with repeat exposure to inhaled endotoxin. *Am J Physiol Lung Cell Mol Physiol* 285: L1337–L1344, 2003.
10. **Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, and Liu AH.** Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 355: 1680–1683, 2000.
11. **Gerhold K, Blumchen K, Bock A, Seib C, Stock P, Kallinich T, Lohning M, Wahn U, and Hamelmann E.** Endotoxins prevent murine

IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 110: 110–116, 2002.
12. **Holt PG.** Dendritic cell ontogeny as an aetiological factor in respiratory tract diseases in early life. *Thorax* 56: 419–420, 2001.
13. **Hsieh TH, Yu CP, and Oberdorster G.** Deposition and clearance models of Ni compounds in the mouse lung and comparisons with the rat models. *Aerosol Sci Technol* 31: 358–372, 1999.
14. **Jain VV, Businga TR, Kitagaki K, George CL, O'Shaughnessy PT, and Kline JN.** Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure. *Am J Physiol Lung Cell Mol Physiol* 285: L1137–L1146, 2003.
15. **Jung K, Ha Y, Ha SK, Han DU, Kim DW, Moon WK, and Chae C.** Antiviral effect of *Saccharomyces cerevisiae* β-glucan to swine influenza virus by increased production of interferon-γ and nitric oxide. *J Vet Med B Infect Dis Vet Public Health* 51: 72–76, 2004.
16. **Justice JP, Crosby J, Borchers MT, Tomkinson A, Lee JJ, and Lee NA.** CD4+ T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. *Am J Physiol Lung Cell Mol Physiol* 282: L1066–L1074, 2002.
17. **Kolls JK and Linden A.** Interleukin-17 family members and inflammation. *Immunity* 21: 467–476, 2004.
18. **Kwon SS, Kim N, and Yoo TJ.** The effect of vaccination with DNA encoding murine T-cell epitopes on the Der p 1 and 2 induced immunoglobulin E synthesis. *Allergy* 56: 741–748, 2001.
19. **Legge KL and Braciale TJ.** Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* 18: 265–277, 2003.
20. **Lorenz E, Jones M, Wohlford-Lenane C, Meyer N, Frees KL, Arbour NC, and Schwartz DA.** Genes other than TLR4 are involved in the response to inhaled LPS. *Am J Physiol Lung Cell Mol Physiol* 281: L1106–L1114, 2001.
21. **Merchant JA, Naleway L, Svendsen R, Kelly M, Burmeister F, Stromquist M, Taylor D, Thorne S, Reynolds J, Sanderson T, and Chrischilles EA.** Asthma and farm exposures in a cohort of rural Iowa children. *Environ Health Perspect* 113: 350–356, 2005.
22. **Metwali N, Thorne PS, Kolegraf K, and O'Neill M.** Enzyme-linked immunosorbent assay specific for (1–6) branched, (1–3)-β-D-glucan detection in environmental samples (abstract). *Am J Resp Crit Care Med* 2: A814, 2005.
23. **Mucida DS, Rodriguez D, Keller AC, Gomes E, Menezes JS, de Faria AM, and Russo M.** Decreased nasal tolerance to allergic asthma in mice fed an amino Acid-based protein-free diet. *Ann NY Acad Sci* 1029: 361–365, 2004.
24. **Neaville WA, Tisler C, Bhattacharya A, Anklam K, Gilbertson-White S, Hamilton R, Adler K, Dasilva DF, Roberg KA, Carlson-Dakes KT, Anderson E, Yoshihara D, Gangnon R, Mikus LD, Rosenthal LA, Gern JE, and Lemanske RF Jr.** Developmental cytokine response profiles and the clinical and immunologic expression of atopy during the first year of life. *J Allergy Clin Immunol* 112: 740–746, 2003.
25. **Nelson DJ and Holt PG.** Defective regional immunity in the respiratory tract of neonates is attributable to hyporesponsiveness of local dendritic cells to activation signals. *J Immunol* 155: 3517–3524, 1995.
26. **Palmqvist M, Cui ZH, Sjostrand M, Linden A, and Lotvall J.** Reduced late asthmatic response by repeated low-dose allergen exposure. *Eur Respir J* 17: 872–880, 2001.
27. **Rautava S, Ruuskanen O, Ouwehand A, Salminen S, and Isolauri E.** The hygiene hypothesis of atopic disease—an extended version. *J Pediatr Gastroenterol Nutr* 38: 378–388, 2004.
28. **Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, Carr D, Schierl R, Nowak D, and von Mutius E.** Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 358: 1129–1133, 2001.
29. **Riffo-Vasquez Y, Spina D, Thomas M, Gilbey T, Kemeny DM, and Page CP.** The role of CD23 on allergen-induced IgE levels, pulmonary eosinophilia and bronchial hyperresponsiveness in mice. *Clin Exp Allergy* 30: 728–738, 2000.
30. **Rodriguez D, Keller AC, Faquim-Mauro EL, de Macedo MS, Cunha FQ, Lefort J, Vargaftig BB, and Russo M.** Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 171: 1001–1008, 2003.
31. **Russo M, Nahori MA, Lefort J, Gomes E, de Castro Keller A, Rodriguez D, Ribeiro OG, Adriouch S, Gallois V, de Faria AM, and Vargaftig BB.** Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* 24: 518–526, 2001.
32. **Schenker MB, Christiani D, Cormier Y, Dimich-Ward H, Doekes G, Dosman J, Douvwes J, Dowling K, Enarson D, Green F, Heederik D, Husman K, Kennedy S, Kullman G, LaCasse Y, Lawson B, Malmberg P, May J, McCurdy S, Merchant J, Myers J, Nieuwanhuijsen M, Olenchock S, Saiki C, Schwartz D, Seiber J, Thorne P, Wagner G, White N, Xu X, and Chan-Yeung M.** Respiratory health hazards in agriculture. *Am J Respir Crit Care Med* 158: S1–S76, 1998.
33. **Singh RR, Hahn BH, and Sercarz EE.** Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J Exp Med* 183: 1613–1621, 1996.
34. **Steerenberg PA, van Dalen WJ, Withagen CE, Dormans JA, and van Loveren H.** Optimization of route of administration for coexposure to ovalbumin and particle matter to induce adjuvant activity in respiratory allergy in the mouse. *Inhal Toxicol* 15: 1309–1325, 2003.
35. **Steinman RM, Hawiger D, and Nussenzweig MC.** Tolerogenic dendritic cells. *Annu Rev Immunol* 21: 685–711, 2003.
36. **Strachan DP.** Hay fever, hygiene, and household size. *BMJ* 299: 1259–1260, 1989.
37. **Suzuki Y, Adachi Y, Ohno N, and Yadomae T.** Th1/Th2-Balancing immunomodulating activity of gel-forming (1–3)-β-glucans from fungi. *Biol Pharm Bull* 24: 811–819, 2001.
38. **Thorne PS.** Inhalation toxicology models of endotoxin- and bioaerosol-induced inflammation. *Toxicology* 152: 13–23, 2000.
39. **Thorne PS, Kiekhaefer MS, Whitten P, and Donham KJ.** Comparison of bioaerosol sampling methods in barns housing swine. *Appl Environ Microbiol* 58: 2543–2551, 1992.
40. **Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ Jr, and Zeldin DC.** Endotoxin exposure is a risk factor for asthma: the National Survey of Endotoxin in US Housing. *Am J Respir Crit Care Med* 172: 1371–1377, 2005.
41. **Todd BE and Buchan RM.** Total dust, respirable dust, and microflora toxin concentrations in Colorado corn storage facilities. *Appl Occup Environ Hyg* 17: 411–415, 2002.
42. **Tschernig T, Debertin AS, Paulsen F, Kleemann WJ, and Pabst R.** Dendritic cells in the mucosa of the human trachea are not regularly found in the first year of life. *Thorax* 56: 427–431, 2001.
43. **Tulic MK, Holt PG, and Sly PD.** Modification of acute and late-phase allergic responses to ovalbumin with lipopolysaccharide. *Int Arch Allergy Immunol* 129: 119–128, 2002.
44. **Tulic MK, Wale JL, Holt PG, and Sly PD.** Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 22: 604–612, 2000.
45. **Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, and Wouters EF.** Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. *Am J Respir Cell Mol Biol* 26: 152–159, 2002.
46. **Von Garnier C, Filgueira L, Wikstrom M, Smith M, Thomas JA, Strickland DH, Holt PG, and Stumbles PA.** Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. *J Immunol* 175: 1609–1618, 2005.
47. **Von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S, Waser M, and Nowak D.** Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 30: 1230–1234, 2000.
48. **Wills-Karp M, Santeliz J, and Karp CL.** The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 1: 69–75, 2001.
49. **Yang LP, Byun DG, Demeure CE, Vezzio N, and Delespesse G.** Default development of cloned human naive CD4 T cells into interleukin-4- and interleukin-5-producing effector cells. *Eur J Immunol* 25: 3517–3520, 1995.

Public Health Implications of the Microbial Pesticide *Bacillus thuringiensis*: An Epidemiological Study, Oregon, 1985–86

MARGARET GREEN, MD, MICHAEL HEUMANN, MPH, ROBERT SOKOLOW, BS, MBA, LAURENCE R. FOSTER, MD, MPH, RICHARD BRYANT, MD, AND MICHAEL SKEELS, PHD, MPH

Abstract: *Bacillus thuringiensis* var. *kurstaki* (B.t.-k) is a microbial pesticide which has been widely used for over 30 years. Its safety for a human population living in sprayed areas has never been tested. Surveillance for human infections caused by B.t.-k among Lane County, Oregon residents was conducted during two seasons of aerial B.t.-k spraying for gypsy moth control. *Bacillus* isolates from cultures obtained for routine clinical purposes were tested for presence of *Bacillus thuringiensis* (B.t.). Detailed clinical information was obtained for all B.t.-positive patients. About 80,000 people lived in the first year's spray area, and 40,000 in the second year's area. A total of 55 B.t.-positive cultures were identified. The cultures had been taken from 18 different body sites or fluids. Fifty-two (95 percent) of the B.t. isolates were assessed to be probable contaminants and not the cause of clinical illness. For three patients, B.t. could neither be ruled in nor out as a pathogen. Each of these three B.t.-positive patients had preexisting medical problems. The level of risk for B.t.-k and other existing or future microbial pesticides in immunocompromised hosts deserves further study. (*Am J Public Health* 1990; 80:848–852.)

Introduction

Bacillus thuringiensis var. *kurstaki* (B.t.-k) is a microbial pesticide which has been used for large scale pest eradication programs in populated areas for more than 30 years. Microbial pesticides are bacteria or viruses that are toxic or infectious to the target pest, usually an insect, but are considered to be harmless to mammals and most other non-target species. In the United States, these programs have been conducted primarily in the Northeast for control of lepidopterous pests, particularly gypsy moth, spruce budworm, and tussock moth infestations. Greater than one million pounds of this pesticide are applied annually in the United States alone.[1] In addition, the bacterium is widely used in commercial landscaping and is sold retail to gardeners for pest control.

Despite its wide use, particularly its aerial application over populated areas, B.t.-k has never been studied epidemiologically to assess its potential for causing human infection. Immunocompromised persons are probably the group most likely to be at risk of infection by a bacterium otherwise harmless to humans.

Bacillus thuringiensis (B.t.) is a gram-positive, spore-forming, facultative soil saprophyte, distinguished by the presence of a crystalline parasporal inclusion.[2,3] Because B.t.-k is not considered to be a mammalian pathogen it has been exempted from pre-harvest and post-harvest restrictions placed upon raw agricultural commodities.[4] Its cell size, 3.0–5.0 × 1.0–1.2 μm, makes it a respirable particle; the spores and toxin crystals of the commercial product are even smaller.[2]

Bacillus thuringiensis is distinguished from *Bacillus cereus* by the presence of a bipyramidal parasporal inclusion body or toxin crystal, which contains delta endotoxin. The toxin crystal is a plasmid-encoded protoxin that is activated under alkaline conditions such as are found in the gut of the gypsy moth larva. The mechanism of action of the pesticide in lepidopteran larvae is (Na,K)-ATPase inhibition, gut paralysis, gut necrosis and consequent cessation of feeding. In addition, the ingested spores may, after germination, result in larval septicemia.[2,5,6] The commercial formulations contain spores and toxin crystals, without any significant component of vegetative cells.

There is only one case of human disease associated with B.t.-k recorded in the medical literature. This occurred after a previously healthy 18-year-old farmer splashed a commercial product into his eye. He was treated with antibiotic ointment. Three days later, when the eye was still irritated, he was treated with a corticosteroid ointment. Ten days after the accident, a corneal ulcer was discovered and was successfully treated with subconjunctival injections of gentamicin and cefazolin sodium. *Bacillus thuringiensis* was cultured from the ulcer.[7,8]

Bacillus thuringiensis has been the subject of many animal and a few human experiments. In one study, 18 human subjects ingested one gram of a commercial B.t.-k product in capsules daily for five days. Five subjects also inhaled 100 mg of the powder daily for five days. No adverse health effects were noted on physical, laboratory, or roentgenologic examination. Laboratory examination did not include cultures of any site.[9]

Virtually all the animal experimentation has been done by agricultural laboratories. Most experiments have used rodents, but some assessed effects on non-target species of agricultural significance, such as farm animals and bees. Most have been done with small numbers of animals, usually without controls, and have shown B.t.-k to be harmless.[10,11]

In reviewing the *Bacillus thuringiensis* literature, distinctions must be made among the subtypes. *Bacillus thuringiensis* var. *israelensis*, for example, used for black fly and mosquito control, appears to be more toxic to mammals than B.t.-k, and has been implicated in a case of human cellulitis.[12] *Bacillus thuringiensis* var. *thuringiensis* has an exotoxin as well as the delta endotoxin, and has been cultured from multiple internal organs of animals autopsied after feeding studies.[10]

Some studies of B.t.-k have also shown pathogenicity of varying degrees. One experiment demonstrated that intraperitoneal injections of large doses of B.t.-k grown in culture caused death in seven out of 10 guinea pigs. Using *B.*

From the Oregon State Health Division, Portland (Green, Foster, Heumann, Sokolow, Skeels), and the Oregon Health Sciences University, Portland (Bryant). Address reprint requests to Laurence R. Foster, MD, MPH, Oregon Health Division, Office of Health Status Monitoring, 1400 S.W. Fifth Avenue, Portland, OR 97201. This paper, submitted to the Journal April 11, 1989, was revised and accepted for publication December 22, 1989.

cereus, the same procedure resulted in the death of five out of five guinea pigs. Needle trauma was ruled out as the cause of death, because none of five guinea pigs that were injected with *Bacillus subtilis* in the same experiment died.[9] B.t.-k and *B. cereus* were shown to persist in blood more than 48 but less than 72 hours following intraperitoneal infection[9]; inhalation, cutaneous injection, topical application to skin abrasions, and intragastric application all yielded no adverse effects in rodents.[9]

There is a single report in the agricultural literature of a fatal, naturally acquired, animal infection attributed to *Bacillus thuringiensis*, variety unknown. This was a case of bovine mastitis, presumably with secondary septicemia.[13]

Background of Present Study

In the summer of 1984, the Oregon Department of Agriculture discovered an infestation of gypsy moths in Lane County, located in the central portion of western Oregon and with a population of 260,000. The infestation included egg masses within the city limits of Eugene. In the fall of 1984, the State of California, which constitutes 70 percent of the market for Lane County forest products, imposed a temporary ban on all finished timber and other wood products from Lane County because of the gypsy moth infestation, and Lane County was quarantined by the United States Department of Agriculture. The Agricultural and Resource Economics Department of Oregon State University estimated that greater than a billion dollars of household income and of timber production would be lost annually if the ban continued.[14]

The decision was made to use B.t.-k instead of chemical pesticides for gypsy moth eradication. *Bacillus thuringiensis* var. *kurstaki* was sprayed from helicopters from May 1 through mid-June 1985, over an area with a population of approximately 80,000 persons; in May 1986, it was sprayed over an area that partly overlapped the first area and had a total population of about 40,000 persons. The bacterial product was mixed with a non-ionic surfactant spreader-sticker-carrier matrix prior to application; polyethylene is the active ingredient of this product.

Methods

Laboratory Surveillance

A passive surveillance program, enrolling the four largest clinical laboratories in the area, was developed to evaluate cultures obtained from human specimens during spraying periods. The laboratories included three in hospitals and one in an outpatient setting. All cultures obtained for routine clinical purposes during, and for one month after, the spray period that were positive for any *Bacillus* species were subcultured by the four participating laboratories, and sent to the Oregon State Public Health Laboratory to establish whether any were positive for B.t. *Bacillus thuringiensis* was identified by the presence of toxin crystals (parasporal inclusions) in bacterial suspension smears stained with basic fuschsin.[15] Follow-up information was collected for all B.t.-k-positive specimens, to determine date of collection, fluid or body site cultured, age and sex of patient, the relative amount of *Bacillus* present on primary culture, and the relative amount and identification of other microorganisms present.

A non-sprayed community approximately 60 miles from the spray area was used as a control community during the second spray season. *Bacillus* species found in specimens from that community's principal hospital were also referred to the State Laboratory for identification.

During the second spray season, culture plates were opened to ambient air for 60 minutes at bench tops in each of the laboratories, to assess the degree of air contamination, and the likelihood that a specimen could have become contaminated on that basis. In addition, culture plates were streaked with sterile wire loops, and blood culture bottles were injected with sterile water to simulate laboratory procedures and assess the level of laboratory contamination. One set of these laboratory environmental samples was done before the spray period; one was done during the spray period, and a third set was done on a day when the area near the laboratory or homes of laboratory workers had been sprayed.

In addition to laboratory surveillance, telephone complaints received by the Lane County Health Department from members of the public were tabulated and evaluated to determine whether there were observable patterns of clinical disease complaints in the general population.

Case Definitions

Physicians were asked the date of onset of the condition for which the positive *Bacillus* culture was obtained, patient history or physical findings which prompted the culture, therapy, outcome (particularly as its time course related to antibiotic therapy), final diagnoses, and other questions pertinent to the particular patient and clinical setting, such as underlying disease states.

Criteria for deciding that B.t.-k could be rejected as a pathogen were set in advance; one or more of the following conditions had to be met:

- There was no clinical evidence of infection.
- The onset of the condition for which the culture was obtained predated the spray period.
- There was evidence of another pathogen or etiology that adequately explained the disease.
- The condition responded to an antibiotic to which B.t. is resistant.
- There was an apparently negligible quantity of B.t. present. (Quantity present was not used as a criterion if the specimen was taken from a normally sterile site, such as blood or urine, except for a urine specimen from a patient with an indwelling bladder catheter.)

Results

During the 1985 study period, 60 subcultures of *Bacillus* species from patient cultures were received by the State Laboratory; 42 were identified as B.t. In 1986, 35 additional clinical *Bacillus* isolates were received from Lane County, of which 13 were B.t. In 1986, seven specimens were received from the control laboratory in the non-sprayed community; none was identified as B.t. Specimens positive for B.t. were taken from 18 different body sites or fluids, including five sites expected to be sterile, 10 environmentally exposed sites, and three other sites.

Data were combined for the two study periods, since there were no known systematic differences between the two spray periods, other than the size of the population sprayed.

Of the laboratory environmental samples, no *Bacillus* species were found in any of the sterile loop-streaked plates or the simulated blood culture sets from any of the laboratories. Of 24 60-minute plates exposed in the sprayed community during the spray period, B.t. was found on two. One was from the bench top in the work area of an employee whose home had been recently sprayed. Non-B.t. *Bacillus* isolates grew on five environmental plates. No *Bacillus* was

grown from environmental plate samples from the control community.

Telephone calls received by the Lane County Health Department did not reveal any pattern of predominance of any one symptom complex or of involvement of any single organ system. Symptoms were those common to any community, e.g., nausea, headache/dysphoria, rash, angioedema.

Fifty-two (95 percent) of the 55 B.t. specimens received were assessed to be probable contaminants, either of skin or tissue or of the laboratory plates (Table 1). One of these was from a spray project worker who sustained a splash of B.t. mixture to his face and eyes and developed dermatitis, pruritis, burning, swelling and erythema, with conjunctival injection. B.t. was cultured from his conjunctiva. He was treated with steroid cream to eyelid and skin, with total resolution. For the remaining three patients, B.t. could not be ruled out as a pathogen; their case histories follow.

PATIENT 1: B.t. cultured from blood—This patient was a 77-year-old male who initially presented June 7, 1985 with a diagnosis of superior vena caval syndrome. Computerized tomography of the thorax revealed a right mediastinal mass and a right pleural effusion. The patient underwent bronchoscopy and mediastinoscopy. Bronchial brushings, needle aspirations and mediastinal biopsy were negative for malignant cells. The patient was treated on an ambulatory basis with prednisone, 40 mg. p.o. per day, and furosemide to effect a diuresis; there was some improvement as a result of these measures.

Two weeks later, a thin needle aspirate of the mediastinal mass was found positive for malignant cells. The procedure was complicated by a right pneumothorax, and the patient was hospitalized. A chest tube was inserted twice during this hospitalization. The steroid dose was tapered down to 10 mg per day at the time of discharge, June 30, 1985. He remained afebrile throughout this hospitalization, and his white blood count was within normal limits.

On July 14, 1985 the patient was readmitted with a fever which had begun two days previously, and cough productive of mucoid sputum without purulence or hemoptysis. On admission, he was dyspneic with rales in the left lung. The temperature peaked at 38.8°C four hours after admission. The chest X-ray showed a left upper lobe infiltrate and pleural fluid. Blood cultures were obtained, and the patient was started on gentamicin and cephazolin. On the third hospital day, the laboratory reported a gram positive *bacillus* in the blood culture. The blood cultures had been taken from two separate sites, each of which was cultured both aerobically and anaerobically. One of the four, an anaerobic bottle, was turbid at 24 hours, and grew out B.t. on subculture. The other three remained negative on repeated subculture over the following seven days. No sputum or other pulmonary specimen cultures were obtained.

Cephazolin was discontinued and cefaperazone was begun when the B.t. was found. On the fifth hospital day, vancomycin was added, and cefaperazone was discontinued. The patient's temperature reached or exceeded 38°C on 8 of 13 days in the hospital, including a second peak of 38.5°C three days after vancomycin was started. He developed progressive renal failure and eventually expired 13 days after admission. The family refused autopsy. The attending physician felt that the patient had pulmonary sepsis, and that this was the proximate cause of death.

PATIENT 18: B.t. cultured from gallbladder contents—This patient was a 31-year-old mentally retarded female with a spastic hemiplegia and seizure disorder secondary to bilateral subdural hemorrhages suffered in a motor vehicle accident 10 years prior to this admission, her 14th. She had chronic pyuria, treated with trimethoprim-sulfamethoxizole. She first presented April 13, 1985 with a history of intermittent upper abdominal pain over the past several months. Findings were non-specific, but she continued to have intermittent or chronic abdominal pain, and presented May 18, 1985 with continued exacerbation of the pain. She was afebrile. An abdominal ultrasound demonstrated "multiple gallstones associated with either a common hepatic duct or common bile duct stone as well." White blood cell count on admission was 8900, but increased to 14,500 on the first post-operative day. Operative findings were "acute hydrops of the gallbladder with a stone impacted at the base of the cystic duct and a marked inflammatory response surrounding the same." The pathologic diagnosis was "acute gangrenous cholecystitis with cholelithiasis." The patient recovered uneventfully from surgery.

A gallbladder fluid specimen was cultured in broth and on seven plates, five aerobic and two anaerobic. All plates showed no growth, but on the fifth day the broth culture was positive for B.t. No other organisms were recovered. No bacteria were seen on histologic examination of gall bladder tissue.

PATIENT 54: B.t. cultured from an antecubital abscess—This patient was a 25-year-old female seen June 26, 1985 at an emergency room with "an abscess on the right forearm, secondary to injecting with methamphetamine on Friday, June 21." She gave a history of four months of IV drug abuse. On physical examination, the patient was afebrile and there were multiple needle puncture lesions. There was no axillary lymphadenopathy and the lesion was soft. The abscess was incised and a small amount of mixed clot was removed. Assessment was "hematoma vs evolving abscess, right forearm." Twenty colonies of B.t. grew from the wound culture taken at that time.

The patient returned on July 1, 1985, at which time the area around the abscess was found to be indurated. She was started on a seven-day course of erythromycin, with resolution of the abscess.

Discussion

Of 55 cultures from human specimens positive for B.t., in no case could B.t. infection unequivocally be said to be the cause of the disease which prompted the clinician to take the culture. Evaluation on the basis of the described criteria suggested that B.t. infection was not the cause of disease in 52 (95 percent) of the cases. In three cases, B.t. could not be ruled in or ruled out as the causative organism.

The first of these three cases occurred in an elderly, immunocompromised person with underlying lung disease. A pneumonia appeared to have contributed to the cause of death in this patient. Specific identification of the cause of this pneumonia was not possible because cultures of sputum, tracheal aspirates, or lung tissue were not performed. It cannot be retrospectively determined whether this patient's positive blood culture (one of four bottles from one of two sites) represented bacteremia or contamination of the specimen at time of collection. Contamination in the laboratory is unlikely because the culture medium was noted to be turbid at 24 hours, before it had been subcultured or vented to air. The patient's fever and the infiltrate seen on chest x-ray suggest that the patient had an infection; the positive blood

TABLE 1—Role of B.t. and Criteria for Assessment in Patients from Whom B.t. Was Cultured

Patient	Site or Body Fluid Cultured	Criterion Codes	Assessed Role of B.t. in Patient: Contaminant (probable or known)	Assessed Role of B.t. in Patient: Pathogenicity Not Ruled Out
Normally Sterile Sites or Fluids				
1	Blood	—		x
2	Blood	iv	x	
3	Blood	iii	x	
4	Blood		x	
5	Blood	iii	x	
6	Blood	i	x	
7	Blood	iii	x	
8	Blood	iv	x	
9	Blood	iii	x	
10	CSF	i	x	
11	CSF	iii	x	
12	Urine	i	x	
13	Urine	iii/iv/v	x	
14	Urine	ii/iii	x	
15	Urine	iii/iv	x	
16	Bursa, synovial fluid	i/iii	x	
17	Bursa, synovial fluid	iii	x	
18	Gallbladder	—		x
Environmentally-Exposed Sites or Body Fluids				
19	Skin rash	ii/v	x	
20	Skin rash	ii/v	x	
21	Skin rash	ii/iii	x	
22	Skin rash	iii	x	
23	Skin rash	iii/iv/v	x	
24	Skin lesion	ii/iii/v	x	
25	Skin lesion	iii/iv/v	x	
26	Skin lesion	iii/iv/v	x	
27	Skin lesion	iv	x	
28	Sputum	ii/iii/v	x	
29	Sputum	i/ii	x	
30	Sputum	iv/v	x	
31	Sputum	iii/v	x	
32	Sputum	iii	x	
33	Cervix	i	x	
34	Cervix	iii/iv/v	x	
35	Cervix	ii	x	
36	Throat	ii	x	
37	Throat	iii/v	x	
38	Throat	iii	x	
39	Wound	iii/iv	x	
40	Wound	iii/iv	x	
41	Wound	iii	x	
42	Wound	iii/iv	x	
43	Wound	iii	x	
44	Wound	i/v	x	
45	Wound	iii	x	
46	Ear	ii/iii/v	x	
47	Ear	iii/iv/v	x	
48	Stool	iii	x	
49	Stool	i/v	x	
50	Nasal mucosa	ii/iii/v	x	
51	Conjunctiva	iii	x	
52	Conjunctiva	iii	x	
Other Sites				
53	Amputation site bone marrow	v	x	
54	Abscess	—		x
55	Cellulitis, hematoma	iii/iv	x	

Criterion Codes
i. No suggestion of infection.
ii. Condition predates spray period.
iii. Evidence of another pathogen or etiology which adequately explains the disease.
iv. Response to antibiotic to which B.t. is resistant.
v. Apparently negligible quantity present.

culture suggests that it might have been caused by B.t. However, the observations that three of four blood cultures were negative and that the patient failed to respond to antibiotics to which the B.t. was susceptible suggest that the pulmonary infection may have been caused by a different organism.

In the second case, B.t. was grown from one of eight cultures of gall bladder fluid collected at time of cholecystectomy for acute gangrenous cholecystitis with cholelithiasis. The only evidence that B.t. might have been causing infection in this case is the positive culture and the absence of other organisms on culture. Evidence against infection includes the observation that only one of eight cultures was positive, and that it was not positive until five days after collection. The patient's lack of fever and the negative histologic examination of the gall bladder for bacteria also argue against infection.

In the third case, B.t. was grown from a possible abscess at an injection site in an IV drug user. The B.t. could have been responsible for this localized infection, but it could also have been a skin or wound contaminant, or it could have colonized an abscess caused by another organism.

In evaluating whether B.t. played a pathogenic role in the other 52 cases, it should be noted that specimens received were from 18 different body sites or fluids. This suggests that this *Bacillus* is usually appearing as a contaminant or commensal, rather than a pathogen, since there is no consistent pattern of disease associated with its presence.

More than three times as many cultures were positive for B.t. in the first year of spraying than in the second year, although the population living in sprayed areas in the first year was only twice as large as in the second year. In the first year, one participating laboratory was at the spray boundary, two were within 0.2 miles of the boundary, and one was 1.4 miles away. In the second year, the closest laboratory was one mile from the spray boundary, while the other three were 1.4 miles, 2.0 miles, and 3 miles away, respectively. This suggests an increased potential for contamination of cultures in the laboratory during the first year compared to the second. The minimal environmental sampling performed, however, does not permit evaluation of this possibility.

The criteria used to assess whether B.t. played a pathogenic role in these other 52 patients were deliberately conservative. Nevertheless, it is possible that B.t.-caused disease may have been missed. B.t. as an opportunist may have exacerbated existing disease, and could have been acting as a co-pathogen or synergist in cases where there was another pathogen or etiology which adequately explained the disease.

The criterion that the condition responded to an antibiotic to which B.t. is resistant raises some theoretical questions. One is whether B.t. could cause self-limiting infections such that the antibiotic administered is irrelevant. A second is that the toxin crystal could cause disease without infection. However, there is experimental evidence to indicate that the toxin crystal of B.t.-k does not produce disease in mammals.[16] Protoxin activation of the toxin crystal requires a pH higher than physiologic mammalian pH; but such alkaline conditions could obtain in microenvironments, such as urine in the presence of urea-splitting *Proteus* spp., a gastrointestinal tract therapeutically treated with antacids, or in the disease state achlorhydria. Portions of the normal gastrointestinal tract, such as the contents of the duodenum and the gallbladder, are also alkaline. Finally, it is known that *Bacillus thuringiensis* var. *israelensis* readily loses its plas-

mid that codes for the toxin crystal, and that resistance to penicillin and cephalosporin antibiotics is lost with the loss of the plasmid; therefore, it is possible that B.t.-k could be rendered sensitive to antibiotics by plasmid loss.[12,19,20]

Many of the complaints from the public received by the Lane County Health Department were related to skin rashes, angioedema, eye irritation, and respiratory involvement. It could be argued that these symptoms are more consistent with disease caused by the insect itself than B.t.-k. There have been reports of major outbreaks of dermatitis in the Northeastern United States involving thousands of persons exposed to gypsy moth larvae. Dermatitis in these outbreaks was attributed to irritation by the setae or hairlike projections of the gypsy moth caterpillar. Associated symptoms of workers exposed to the larvae included rhinitis, irritation of the eyes, and dyspnea. Wheal and flare reactions have occurred in response to scratch tests in persons with a history of exposure to gypsy moths.[20,21] Eighty ng/organism of histamine have been extracted from these setae.[17,18]

Bacillus thuringiensis var. *kurstaki* has a remarkable safety record in view of its wide use by gardeners, in agriculture, and for major pest eradication projects such as the one undertaken in Lane County. However, *Bacillus* species found in laboratory cultures are usually considered to be contaminants and are not identified, other than to rule out pathogens or opportunists, such as *Bacillus anthracis*, *B. cereus*, or *B. subtilis*. Identification of the toxin crystal involves a specialized technique, not usually indicated for purposes of medical care.[15] Therefore, B.t. disease could have been missed in persons who had contact with the pesticide in other sprayed areas, simply because the *Bacillus* was not known to be B.t. and was interpreted as a contaminant.

In the 30 years during which B.t. has been in widespread use, there has been an increasing proportion of persons in any community who are immunocompromised on some basis. At the same time, the medical community has become more reluctant to label any bacterium as absolutely non-pathogenic to humans. Perhaps the best example of a pathogen once thought to be non-pathogenic to humans is *Serratia marcescens*, now recognized as one of the 15 most frequently isolated pathogens in association with nosocomial infectious diseases in hospitals.[22]

Bacillus species in general can no longer be dismissed as harmless commensals or skin or air contaminants. The current concept is that any isolate should be considered in its clinical context to decide whether it is a pathogen.[23]

This study raises a fundamental issue to be considered by agencies responsible for regulating the use of microorganisms for pest control. These microorganisms may have potential for causing disease in immunocompromised persons. Therefore, such individuals should be advised on how to use biopesticides and how to protect themselves from undue exposure in areas where they are used. Introductions of other biological agents being considered for pest control should occur only after their safety for the seriously compromised host is evaluated. This is important in light of the increasing potential usefulness of microorganisms for pest control made possible by the new technologies of genetic engineering.[24–26]

REFERENCES

1. DeLucca AJ II, Simonson JG, Larson AD: *Bacillus thuringiensis* distribution in soils of the United States. Can J Microbiol 1981; 27:865–870.
2. Deacon JW: Microbial control of pests: use of bacteria. *In:* Deacon, JW (ed): Microbial Control of Plant Pests and Diseases. Washington, DC: American Society for Microbiology, 1983; 8–18.
3. Doyle RJ, Keller KF, Ezzell JW: *Bacillus*. *In:* Lennette EH (ed): Manual of Clinical Microbiology, Fourth Edition. Washington, DC: American Society for Microbiology, 1985; 211–215.
4. *Bacillus thuringiensis* var. *kurstaki*. *In:* Berg, *et al*, (eds): Farm Chemicals Handbook '86. Willoughby, OH: Meister Publishing Co, 1986; Ch25.
5. Aronson AI, Beckman W, Dunn P: *Bacillus thuringiensis* and related insect pathogens. Washington, DC: American Society for Microbiology, Microbiological Reviews 1986; 1–24.
6. English LH, Cantley LC: Delta endotoxin is a potent inhibitor of the (Na,K)-ATPase. J Biol Chem 1986; 261:1170–1173.
7. Samples JR, Buettner H: Corneal ulcer caused by a biologic insecticide (*Bacillus thuringiensis*). Am J Ophthalmol 1983; 95:258–260.
8. Samples JR, Beuttner H: Ocular infection caused by a biological insecticide. J Infect Dis 1983; 148:614.
9. Fisher R, Rosner L: Toxicology of the microbial insecticide, Thuricide. Agriculture Food Chem 1959;7:686–688.
10. Forsberg CW: *Bacillus thuringiensis*: Its Effects on Environmental Quality. Ottowa: National Research Council of Canada, 1976; 56–69.
11. Cantwell GE, Knox DA, Lehnert T, Michael AS: Mortality of the honey bee, *Apis mellifera*, in colonies treated with certain biological insecticides. J Invertebr Pathol 1966; 8:228–233.
12. Warren RE, Rubenstein D, Ellar DJ, Kramer JM, Gilbert RJ: *Bacillus thuringiensis* var. *Israelensis*: protoxin activation and safety. Lancet 1984; 1:678–679.
13. Gordon RE: Some taxonomic observations on the genus *Bacillus*. *In:* Briggs JD (ed): Biological Regulation of Vectors: The saprophytic and aerobic bacteria and fungi. US DEW pub. no. NIH-77-1180. Washington, DC: Govt Printing Office, 1977; 67–82.
14. Stringham TK, Radtke H, Nelson AG: A preliminary assessment of the economic impact of the gypsy moth infestation in Lane County. Corvallis, Oregon: Oregon State University, 1985.
15. Harmon SM: *Bacillus cereus*. *In:* Bacteriological Analytical Manual, 6th Edition. US Food and Drug Administration. Arlington, VA: Association of Official Analytical Chemists, 1984; 16.07.
16. Thomas WE, Ellar DJ: *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: Effects on insect and mammalian cells *in vitro* and *in vivo*. J Cell Sci 1983; 60:181–195.
17. Gonzalez JM Jr, Brown BJ, Carlton BC: Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *Bacillus thuringiensis* and *B. cereus*. Proc Natl Acad Sci (USA) 1982; 79:6951–6955.
18. Ruhfel RE, Robillard NJ, Thorne CB: Interspecies transduction of plasmids among *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. J Bacteriol 1984; 157:708–711.
19. Aber R, DeMelfi T, Gill T, Healey B, McCarthy MA, Oswell N, Ruhig W, Speziale H, Witte EJ: Rash illness associated with gypsy moth caterpillars—Pennsylvania. MMWR 1982; 31:169.
20. Shama SK, Etkind PH, Odell TM, Canada AT, Finn AM, Soter NA: Gypsy-moth-caterpillar dermatitis. N Engl J Med 1982; 306:1300–1302.
21. Tuthill RW, Canada AT, Wilcock K, Etkind PH, O'Dell TM, Shama SK: An epidemiologic study of gypsy moth rash. Am J Public Health 1984; 74:799–803.
22. Acar JF: *Serratia marcescens* infections. Infect Control 1986; 7:273–278.
23. Tuazon CU, Murray HW, Levy C, Solny MN, Curtin JA, Sheagren JN: Serious infections from *Bacillus sp*. JAMA 1979; 241:1137–1140.
24. Fox JL: Anticipating deliberate release. Am Soc Microbiol News 1985; 51:619–620.
25. Trewhitt J, Bluestone M: Agrichemical firms turn to genetic engineering. Chem Week 1985:34–40.
26. Vidaver AK: Plant-associated agricultural applications of genetically engineered microorganisms: projections and constraints. *In:* Recombinant DNA Technical Bulletin. National Institutes of Health, 1985; US Department of Health and Human Services 1985; 8:97–102.

The Relationship of Skin Test Positivity, High Serum Total IgE Levels, and Peripheral Blood Eosinophilia to Symptomatic and Asymptomatic Airway Hyperresponsiveness

DÉSIRÉE F. JANSEN, BERT RIJCKEN, JAN P. SCHOUTEN, JAN KRAAN, SCOTT T. WEISS, WIM TIMENS, and DIRKJE S. POSTMA

Departments of Epidemiology and Statistics, University of Groningen, Pulmonology and Pathology, University Hospital of Groningen, The Netherlands; and Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

The relationships of skin test positivity, high serum total IgE levels (> 100 kU/L), and peripheral blood eosinophilia (≥ 275 cells/μl) to symptomatic (either chronic cough, chronic phlegm, bronchitis episodes, dyspnea, wheeze, or asthma) and asymptomatic bronchial hyperresponsiveness (BHR) were studied cross-sectionally in 620 adult subjects who participated in the Vlagtwedde–Vlaardingen Study of 1989 and 1990. Eosinophilia (OR = 2.06, 95% CI = 1.28 to 3.31) and skin test positivity (OR = 1.66, 95% CI = 1.02 to 2.71) were both significantly associated with BHR independent of age, sex, smoking, and urban area of residence. High serum total IgE levels were not associated with BHR (OR = 1.29, 95% CI = 0.81 to 2.03). Separate analyses for symptomatic and asymptomatic subjects showed that the higher risk of BHR with skin test positivity applied only to symptomatic subjects (OR = 5.78, 95% CI = 1.63 to 20.51), independent of eosinophilia and high serum total IgE levels. The higher risk of BHR with eosinophilia was not different between symptomatic and asymptomatic subjects, and independent of skin test positivity and high serum total IgE levels. The results of this study show that, in the general adult population, eosinophilia is associated with BHR both in symptomatic and asymptomatic persons, whereas skin test positivity is associated with BHR only in symptomatic subjects. Jansen DF, Rijcken B, Schouten JP, Kraan J, Weiss ST, Timens W, Postma DS. The relationship of skin test positivity, high serum total IgE levels, and peripheral blood eosinophilia to symptomatic and asymptomatic airway hyperresponsiveness.

AM J RESPIR CRIT CARE MED 1999;159:924–931.

Bronchial hyperresponsiveness (BHR), the exaggerated airway narrowing in response to nonspecific stimuli, is a common characteristic of asthma. However, BHR is also present in 19 to 62% of subjects without respiratory symptoms in the general population (1). The mechanisms underlying asymptomatic BHR are still unclear, yet it is known that in asymptomatic subjects BHR is a risk factor for asthma and chronic obstructive pulmonary disease (COPD) (2–5). Furthermore, most subjects with BHR who develop respiratory symptoms appear to be atopic or have a positive family history of atopy (3, 4). Thus, a central question is whether atopy helps to distinguish asymptomatic from symptomatic BHR.

Atopy is a process mediated by immunoglobulin E (IgE) (6). Mast cells in the bronchial wall are activated by IgE and as a consequence release mediators that may cause BHR directly (histamine, prostaglandins, and leukotrienes) or indirectly (interleukin-4 [IL-4] and tumor necrosis factor α [TNF-α]). IL-4 stimulates B cells to produce IgE, which maintains mast cell activation. Further, IL-4 and TNF-α enable eosinophils to migrate from the vessels into the bronchial mucosa by upregulation of vascular adhesion molecules (6, 7). Eosinophils, once activated, release mediators, which damage the epithelium and cause BHR due to increased permeability (6, 8, 9). However, increased serum total IgE levels and peripheral blood eosinophil counts are neither closely related nor exclusively present in atopic individuals. Serum total IgE levels are also increased in nonatopic smokers, and peripheral blood eosinophils are also elevated in parasitic infections and in certain neoplasms (10). Thus, skin test responses, serum total IgE level, and number of peripheral blood eosinophils are independent features of a common underlying mechanism.

The use of different measures of atopy in different studies has complicated comparisons of the results. However, BHR is clearly more prevalent among subjects with positive skin prick tests (11, 12), increased serum total or specific IgE levels (13, 14), and peripheral blood eosinophilia (15–17). It is unknown which of these atopy measures most strongly correlates with BHR. To determine which atopy-related factor best predicts BHR, we performed cross-sectional analyses on the relation-

(*Received in original form April 2, 1998 and in revised form November 3, 1998*)

Supported by the Netherlands Asthma Foundation, Grant 93.66.

Correspondence and requests for reprints should be addressed to Prof. dr. D. S. Postma, Department of Pulmonary Diseases, University Hospital Groningen, P.O. Box 30.001, NL-9700 RB Groningen, The Netherlands.

Am J Respir Crit Care Med Vol 159. pp 924–931, 1999
Internet address: www.atsjournals.org

ship between BHR and positive skin prick tests, increased serum total IgE levels, and peripheral blood eosinophilia. As we are interested in differences in mechanisms underlying asymptomatic BHR and symptomatic BHR in particular, we also determined these relationships in subjects with and without respiratory symptoms.

METHODS

The current analyses made use of data from the 1989 and 1990 survey of the Vlagtwedde–Vlaardingen Study, a longitudinal population study that was started in 1965 to determine risk factors for COPD. The selection of the study population has been described previously (18, 19). After baseline measurements in 1965 and 1967 in Vlagtwedde and in 1965 and 1969 in Vlaardingen, a follow-up survey was organized every 3 yr. In the first two baseline surveys a histamine provocation test was performed in a 25% random sample from the study population. From the third survey on, the strategy has been to select those subjects for histamine testing who had performed the test in prior surveys. As a result from this strategy 808 of the 2,553 participants (age 35 to 80 yr) in the surveys of 1989 and 1990 underwent a histamine provocation test. These subjects make up the group included in this cross-sectional study. Both surveys were carried out during the month of October.

Information on respiratory symptoms, smoking status, age, and sex was collected by the Dutch version of the British Medical Research Council standardized questionnaire (20). Subjects were considered symptomatic if they reported one or more of the following chronic respiratory symptoms: cough or phlegm production on most days or nights for as much as 3 consecutive months each year during winter (referred to as chronic cough/chronic phlegm), a period of at least 3 wk in the previous 3 yr with (increased) cough and phlegm (bronchitis episodes), shortness of breath when walking with other persons of the same age on level ground (dyspnea $\geqslant$ grade 3), a wheezing or whistling sound in the chest on most days or nights (persistent wheeze), or attacks of shortness of breath at any time (asthma attacks). Subjects were considered asymptomatic if they reported none of these chronic respiratory symptoms. Subjects were categorized as current smokers, ex-smokers (defined as those who had quit smoking at least 1 mo before the examination), and never-smokers.

Pulmonary function measurements were performed with a water-sealed spirometer (Lode Spirograph D53; Lode Instruments, Groningen, The Netherlands). Measurement of inspiratory vital capacity (IVC) after a deep expiration was followed by measurement of forced expiratory volume in one second (FEV_1). The higher of the values obtained in two technically satisfactory tracings was taken as the baseline measurement as long as the difference between the two IVC values was less than 150 ml and that between the two FEV_1 values was less than 100 ml.

Bronchial responsiveness to histamine was assessed by the method of Tiffeneau as modified by De Vries and coworkers (21), which meets standardization guidelines (22). After baseline measurements of pulmonary function, subjects inhaled nebulized distilled water from a Wiesbaden Doppel inhalator (Lode Instruments, Groningen, The Netherlands). If the IVC and/or the FEV_1 decreased by 10% or more, the test was terminated ($n = 2$). If the IVC or the FEV_1 did not decrease by 10% or more, the test proceeded with the application of sequential aerosols of histamine biphosphate in concentrations of 1, 4, 8, 16, and 32 mg/ml. Each concentration was inhaled for 30 s. After each challenge, two IVC and FEV_1 maneuvers were performed. If, at a given concentration, the IVC or the FEV_1 persistently declined by 10% or more, this particular concentration was considered the threshold value (PC_{10}). The test was terminated with a persistent decrease of 10% or more or after administration of the highest concentration. BHR was defined as a PC_{10} of $\leqslant$ 8 mg/ml histamine (including a $\geqslant$ 10% decrease after distilled water inhalation). Subjects with excessively low levels of pulmonary function ($FEV_1 < 1.5$ L) and those who could not perform a forced expiration were not tested. Subjects suffering from heart disease, hypertension, or acute respiratory infections were also excluded from challenge.

Skin prick tests (ALK Benelux, Woerden, The Netherlands) included six inhalant allergens: house dust mite (*Dermatophagoides pteronyssinus*), mixed grass pollen (meadow foxtail, cocksfoot, meadow fescue, rye grass [perennial], and timothy), mixed tree pollen (alder, birch, and hazel), dog epithelium, cat epithelium, and mold (*Aspergillus fumigatus*). The solvent for the allergens (50% glycerol and 50% aqueous isotone) served as a negative control, and a histamine dihydrochloride solution (3 mg/ml) served as a positive control. Skin prick tests were quantified 15 min after application as the mean value of the longest diameter of the wheal and its perpendicular and were considered positive at $\geqslant$ 3 mm. Skin test positivity was defined as one or more positive skin prick tests.

Before spirometry and skin prick tests were performed, blood samples were taken. Peripheral blood eosinophil counts were estimated with a Technicon-H1 blood cell counter (Bayer AG, Leverkusen, Germany) and were expressed as number of cells per microliter. Peripheral blood eosinophilia was defined as $\geqslant$ 275 cells/μl (23). Total serum IgE concentrations were determined with the CAP system (Pharmacia, Woerden, The Netherlands) and expressed in kU/L. Concentrations below 2 kU/L and above 2,000 kU/L cannot be detected by this system and were represented by 1.99 kU/L and 2,001 kU/L, respectively. High serum total IgE levels were defined as $>$ 100 kU/L.

Data Analyses

Chi-square tests and *t* tests were used to compare the characteristics of subjects with and without respiratory symptoms (24). Logistic regression analyses were performed to determine the relationship between BHR and skin test responses, serum total IgE levels, and peripheral blood eosinophilia (independent of smoking habits, age, gender, and area of residence) (24). Each measure of atopy was dichotomized i.e., the presence or absence of skin test positivity, of a high serum total IgE level, and of peripheral blood eosinophilia. To determine how the various measures of atopy influence each other in relation to BHR, we initially included only one of the three measures of atopy in the regression analyses. We then repeated the analyses, including two measures of atopy and finally all three measures of atopy. We stratified the analyses by respiratory symptoms to determine whether the relationship was the same in symptomatic as in asymptomatic subjects. To test the difference between symptomatic and asymptomatic subjects, we added three interaction terms for each measure of atopy by respiratory symptoms to the pooled analyses (24). Because the presence of respiratory symptoms in the general population is intermittent, we also stratified by "respiratory symptoms in the past" i.e., subjects who never reported respiratory symptoms during the follow-up period from 1965 to 1990 (always asymptomatic), those who reported respiratory symptoms at least once but not at all surveys (intermittently symptomatic), and those who always reported respiratory symptoms (always symptomatic).

All analyses were repeated with bronchial responsiveness, or peripheral blood eosinophil counts, skin test positivity, and serum total IgE levels as continuous variables. Bronchial responsiveness was expressed as the values 1 to 6 for the threshold values 1, 4, 8, 16, 32, and $>$ 32 mg/ml and log-transformed. Only subjects with complete information on all variables were included in the analyses.

RESULTS

Of the 808 subjects available for this study, 691 (85.5%) performed technically satisfactory FEV_1 and IVC measurements. Values on peripheral blood eosinophilia, skin test positivity, or serum total IgE levels were missing for 71 subjects, leaving complete information of 620 subjects for analyses. Respiratory symptoms in the previous 3 yr were reported by 136 (22%) subjects. Subjects with respiratory symptoms were more likely than subjects without respiratory symptoms to be current smokers, to have lower levels of pulmonary function, and to be hyperresponsive to histamine (Table 1). The proportion of subjects who were male and the subjects' mean age were not significantly different in the two groups.

Peripheral blood eosinophilia was detected in 15.3% of all subjects, skin test positivity in 14.7%, and a high serum total IgE level in 16.9%. The prevalences of each atopy measure were similar in symptomatic and asymptomatic subjects. The

TABLE 1

CHARACTERISTICS OF PARTICIPANTS IN THE VLAGTWEDDE–VLAARDINGEN SURVEYS OF 1989 AND 1990 BY RESPIRATORY SYMPTOM STATUS

	Asymptomatic	Symptomatic
n (% men)	484 (58)	136 (60)
Age, yr, mean ± SD	53 ± 10	53 ± 10
Current smokers, n (%)	149 (31)	60 (44)*
Ex-smokers, n (%)	200 (41)	40 (29)†
Nonsmokers, n (%)	135 (28)	36 (27)
FEV_1, % pred, mean ± SD	110 ± 15	103 ± 18‡
FEV_1/VC, %, mean ± SD	75 ± 7	73 ± 9‡
$PC_{10} \leq 8$ mg/ml, n (%)	173 (36)	71 (52)‡
Peripheral blood eosinophilia, n (%)	75 (16)	20 (15)
Skin test positivity, n (%)	71 (15)	20 (15)
Serum total IgE level > 100 kU/L, n (%)	83 (17)	22 (16)

* $p < 0.01$.
† $p < 0.05$.
‡ $p < 0.001$.

overlap among the three measures was small, though the prevalence of atopy defined by any one of the three measures alone was almost the same (Figure 1). Nevertheless, different subjects will be considered atopic when different measures of atopy are applied. At least one positive measure of atopy was observed in 35.6% of the subjects; all three measures were observed in only 1.5%.

Figure 2 shows that the percentage of subjects with BHR increases with higher numbers of peripheral blood eosinophils both among subjects with and without symptoms. The prevalences of BHR without respiratory symptoms were similar among skin test–positive and skin test–negative subjects, whereas the prevalence of BHR with respiratory symptoms was higher among skin test–positive subjects (Figure 3). The prevalences of BHR were similar across quartiles of serum total IgE levels among both asymptomatic and symptomatic subjects, although symptomatic subjects had higher serum total IgE levels (Figure 4).

Logistic Regression for Each Measure of Atopy

Logistic regression analyses for each of the three measures of atopy considered separately with age, sex, smoking status, and area of residence taken into account showed that subjects with peripheral blood eosinophilia or with skin test positivity were more likely to have BHR than subjects in the corresponding negative groups (Table 2). High serum total IgE levels were not associated with BHR. Stratified analyses showed a higher risk of BHR with skin test positivity in symptomatic subjects than in asymptomatic subjects; the difference was of borderline significance ($p = 0.08$). The associations of BHR with pe-

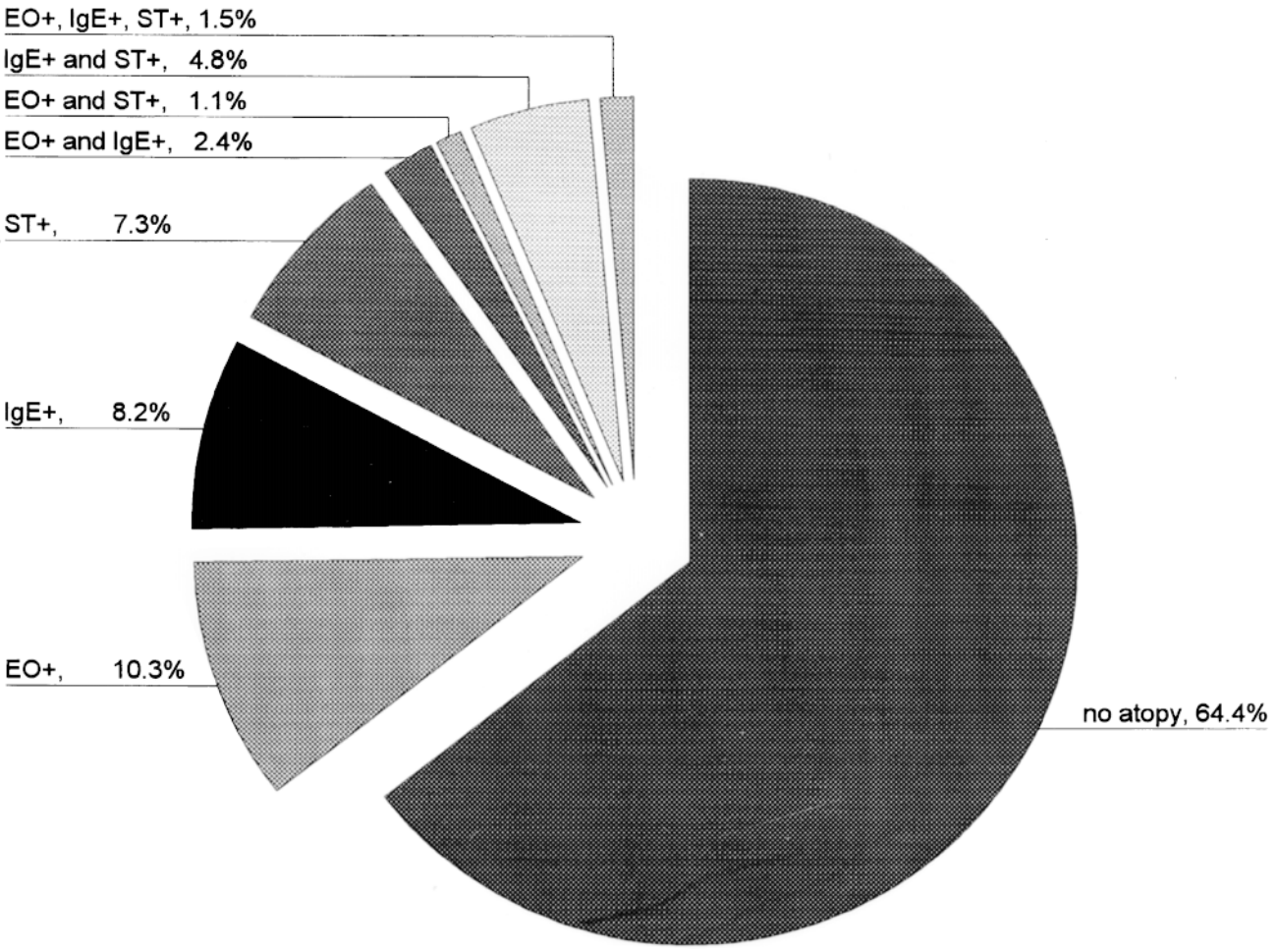


Figure 1. Prevalence of atopy defined by eosinophilia, skin test positivity, and high serum total IgE levels. EO+ = eosinophilia; IgE+ = serum total IgE level > 100 kU/L; ST+ = skin test positivity.

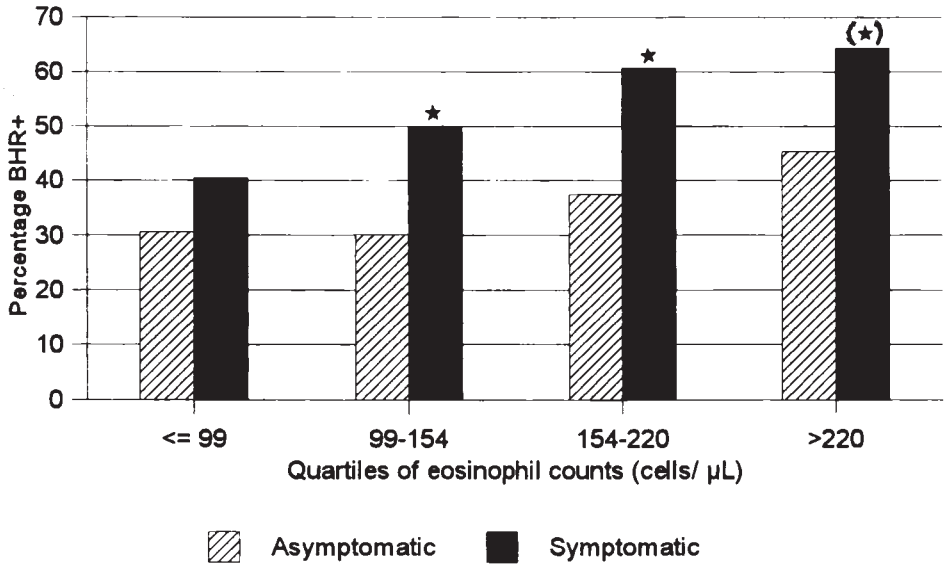


Figure 2. Prevalence of BHR by quartiles of eosinophil counts and respiratory symptoms. *p < 0.05, (*) p = 0.07 compared with asymptomatic.

ripheral blood eosinophilia and high serum total IgE levels were not significantly different for symptomatic versus asymptomatic subjects. Exclusion of the 35 subjects with asthma-like symptoms (e.g., wheeze most days and/or nights or attacks of shortness of breath with wheeze) did not change the observed relationships between measures of atopy and BHR in symptomatic subjects.

Logistic Regression Including Different Combinations of Two Measures of Atopy

To determine whether the various measures of atopy are independently associated with BHR, the analyses were repeated with the inclusion of combinations of two measures. In the total group, the overall risks of BHR assessed in terms of the presence of peripheral blood eosinophilia, skin test positivity, or high serum total IgE level did not change when adjusted for one of the other measures of atopy.

The risks of BHR in symptomatic subjects with skin test positivity or high serum total IgE levels changed when these two measures of atopy were adjusted for each other: the risk with skin test positivity increased from 4.19 (Table 2) to 5.82 (95% confidence interval [CI] = 1.66 to 20.46), whereas the risk with high serum total IgE levels decreased from 0.91 (Table 2) to 0.48 (95% CI = 0.16 to 1.44). In asymptomatic subjects, the risk estimates remained similar after these adjustments.

Logistic Regression Including All Three Measures of Atopy

The changes previously described persisted when all three measures of atopy were combined in the same regression

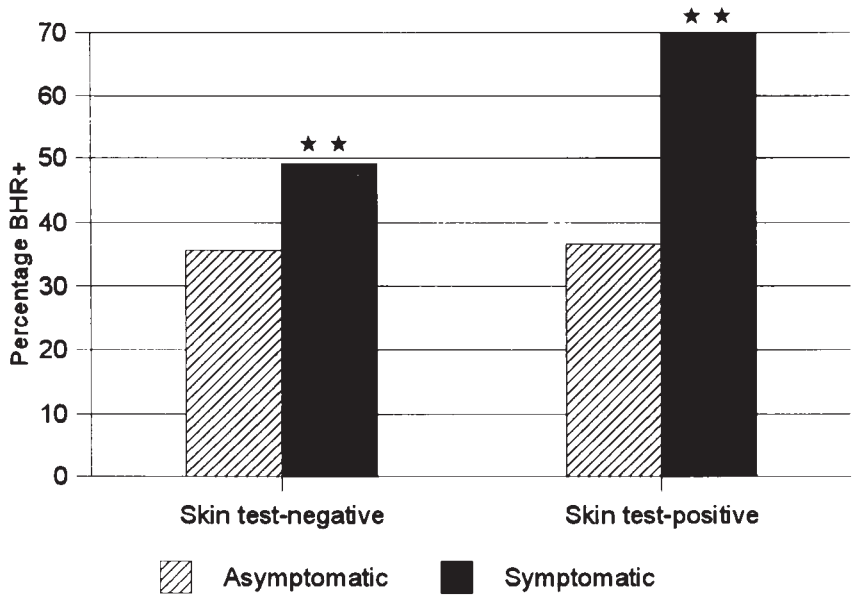


Figure 3. Prevalence of BHR by skin test positivity and respiratory symptoms. **p < 0.01 compared with asymptomatic.

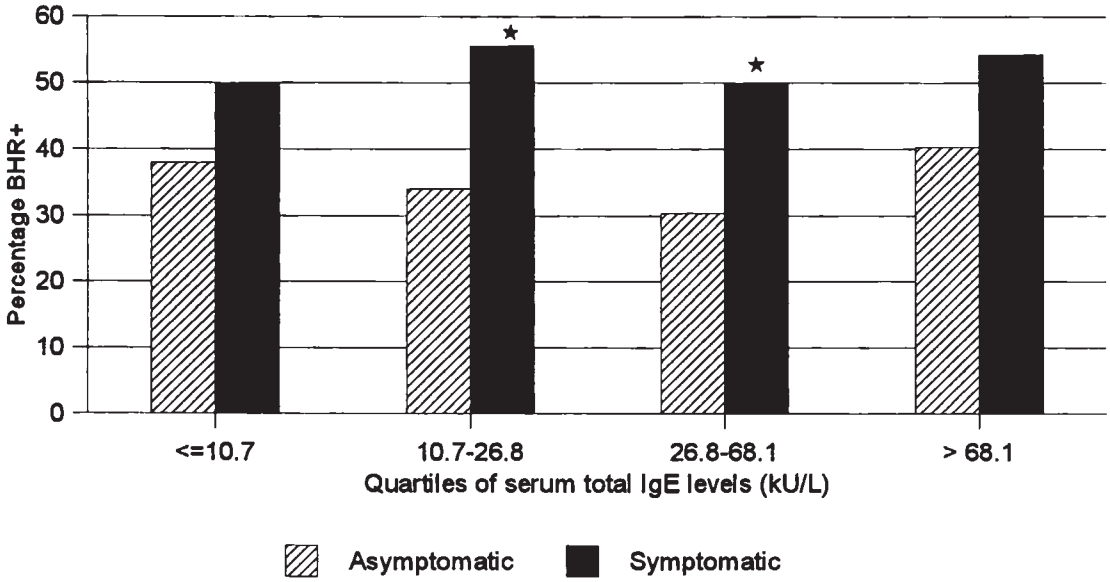


Figure 4. Prevalence of BHR by quartiles of serum total IgE levels and respiratory symptoms. *$p < 0.05$ compared with asymptomatic.

model (Table 3). In symptomatic subjects, skin test positivity was a stronger predictor of BHR than peripheral blood eosinophilia. Serum total IgE levels were not associated with BHR, though levels were almost significantly different between symptomatic and asymptomatic subjects ($p = 0.09$).

A further difference with respect to respiratory symptoms is that among asymptomatic subjects females are more likely to have BHR than males, whereas among symptomatic subjects no difference was observed between the sexes. This difference between the symptomatic and asymptomatic groups was no longer significant after adjustment for FEV_1. Furthermore, smoking at an older age had a synergistic effect on the risk of BHR, as indicated by a significant positive interaction between smoking and age (Table 3).

Finally, we stratified the analyses not only by the presence or absence of respiratory symptoms in the surveys of 1989 and 1990 but also with reference to "respiratory symptoms in the past." As the relations among the three measures of atopy and BHR were not significantly different for subjects who had been intermittently symptomatic in the past but were asymptomatic in the last survey (1989/1990) versus subjects who had been intermittently symptomatic in the past and were symptomatic in the last survey, these groups were pooled and designated as "intermittently symptomatic." Analyses showed that peripheral blood eosinophilia is a risk factor for BHR in subjects who have been intermittently symptomatic but not in subjects who have always been either asymptomatic or symptomatic (Table 4). No changes were observed in the relationships between skin test positivity and BHR and between high serum total IgE levels and BHR when "respiratory symptoms in the past" were taken into account.

Similar relationships were found between the three atopy measures when bronchial responsiveness was analyzed as a continuous variable. Analyses with the continuous measures of the number of peripheral blood eosinophils, the sum of the wheal sizes of the positive skin tests, and the level of serum total IgE yielded results comparable to those from the analyses with the dichotomized measures. Similarly, additional adjustment for pulmonary function (FEV_1) did not change the results. To be sure that the exclusion of subjects who performed technically unsatisfactory lung function measurements (FEV_1 and IVC) caused no selection, we reanalyzed the relationships including the information of this group. The results were similar.

DISCUSSION

The results of this study conducted in a general adult population with a mean age of 53 yr show that peripheral blood eo-

TABLE 2

ODDS RATIOS (95% CI) FOR BHR CALCULATED IN THREE LOGISTIC REGRESSION MODELS FOR EACH ATOPY PARAMETER SEPARATELY*

	OR (95% CI)		
Parameter	Total Group (*n = 620*)	Asymptomatic (*n = 484*)	Symptomatic (*n = 136*)
Eosinophilia	2.06† (1.28–3.31)	2.16† (1.25–3.70)	1.76 (0.64–4.89)
Positive skin tests	1.66‡ (1.02–2.71)	1.31 (0.74–2.30)	4.19‡ (1.36–12.89)
Serum total IgE > 100 kU/L	1.29 (0.81–2.03)	1.47 (0.88–2.47)	0.91 (0.35–2.37)

* All odds ratios are adjusted for age, sex, smoking status, and area of residence. The risks of BHR for the three measures of atopy were not significantly different for symptomatic versus asymptomatic subjects.

† $p < 0.01$.

‡ $p < 0.05$.

TABLE 3

ODDS RATIOS (95% CI) FOR BHR OF THREE DIFFERENT ATOPY PARAMETERS COMBINED IN ONE LOGISTIC REGRESSION MODEL*

	OR (95% CI)		
	Total Group (*n* = 620)	Asymptomatic (*n* = 484)	Symptomatic (*n* = 136)
Atopy measures			
Eosinophilia	2.07† (1.28–3.37)	2.10† (1.22–3.63)	2.00 (0.69–5.75)
Skin test positivity	1.13 (0.63–2.03)	1.12 (0.62–2.01)	5.78† (1.63–20.51)§
Serum total IgE > 100 kU/L	1.40 (0.81–2.41)	1.39 (0.80–2.40)	0.48 (0.16–1.49)
Variables adjusted for			
Respiratory symptoms	1.25 (0.63–2.48)		
Age, yr	1.04† (1.01–1.06)	1.04† (1.01–1.06)	1.04 (0.99–1.09)
Male sex	0.48† (0.31–0.75)	0.47† (0.30–0.74)	1.34 (0.57–3.18)
Current smoker	0.12 (0.01–1.10)	0.15 (0.01–1.97)	0.08 (0.00–7.24)
Ex-smoker	1.56 (0.96–2.53)	1.63 (0.94–2.83)	1.31 (0.46–3.68)
Urban area of residence	0.59† (0.41–0.85)	0.62‡ (0.40–0.94)	0.52 (0.24–1.11)
Interaction terms			
Current smoker × age	1.07† (1.02–1.11)	1.06‡ (1.01–1.12)	1.07 (0.98–1.17)
Symptoms × skin test positivity	4.90‡ (1.30–18.51)		
Symptoms × serum total IgE > 100 kU/L	0.34 (0.10–1.17)		
Symptoms × male sex	2.45‡ (1.04–5.78)		

* All odds ratios are adjusted for age, sex, smoking status, and area of residence.
† $p < 0.01$.
‡ $p < 0.05$.
§ The risk of BHR with skin test positivity is significantly higher among symptomatic than asymptomatic subjects.

sinophilia and skin test positivity significantly predict BHR, independent of each other and of a high serum total IgE level. The relationship between peripheral blood eosinophilia and BHR was the same in asymptomatic subjects and symptomatic subjects, whereas skin test positivity was predictive of BHR in symptomatic subjects only. In contrast, high serum total IgE levels were not associated with BHR in this middle-aged population.

Positive skin test responses, an increased level of serum total IgE, and increased numbers of peripheral blood eosinophils are generally considered to reflect atopy. Earlier results in the Vlagtwedde–Vlaardingen Study showed positive associations among these three measures of atopy (25). Therefore, their relationships with BHR were expected to become weaker when the measures of atopy were adjusted for one another. However, peripheral blood eosinophilia, skin test positivity, and a high serum total IgE level did not influence one another's relationships with BHR, apart from a higher risk of BHR with skin test positivity in symptomatic subjects when adjusted for serum total IgE levels. This result is explained to a large extent by the small overlap between the three measures of atopy (Figure 2). The implication is that skin test positivity, a high serum total IgE level, and peripheral blood eosinophilia are different expressions of the atopic phenotype. This conclusion is in accord with other studies in children (26) and young adults (27), in which many young adults without a positive skin test have had high serum total IgE levels.

One of the important messages of this study is that peripheral blood eosinophilia is associated with an independent increased risk of BHR in asymptomatic and symptomatic individuals. Only two previous studies have determined the relationships between different atopy measures and BHR independent of each other (28, 29). In an adult population including only males, all three measures were significantly, though weakly, associated with increased bronchial responsiveness (28). In another study conducted in a general population, both skin test positivity to cat dander (odds ratio [OR] = 5.5, $p < 0.05$) and high serum total IgE levels (OR = 2.2 per log unit, $p < 0.05$) were strong predictors of BHR, whereas peripheral blood eosinophilia was not related to BHR (29). A

TABLE 4

ODDS RATIOS (95% CI) FOR BHR ACCORDING TO SYMPTOM HISTORY, BASED ON SYMPTOM STATUS IN ALL SURVEYS*

	OR (95% CI)		
	Always Asymptomatic (*n* = 287)	Intermittently Symptomatic (*n* = 295)	Always Symptomatic (*n* = 38)
Eosinophilia	1.04 (0.47–2.30)	3.45† (1.74–6.86)‡	0.58 (0.06–5.69)
Skin test positivity	0.98 (0.41–2.35)	1.61 (0.80–3.23)	22.09 (0.84–580)
Serum total IgE > 100 kU/L	1.55 (0.70–3.45)	0.87 (0.45–1.69)	1.12 (0.11–11.48)

* The allergy parameters are simultaneously included in the model and adjusted for age, sex, smoking status, and area of residence.
† $p < 0.001$.
‡ The risk of BHR for subjects with peripheral blood eosinophilia is significantly higher in the presence of intermittent respiratory symptoms than in the presence of continuous symptoms.

lower number of peripheral blood eosinophils (median, 110 cells/μl versus 154 cells/μl in our population) might explain this lack of relation between BHR and peripheral blood eosinophilia (personal communication). Our study showed a higher risk of BHR in symptomatic and asymptomatic subjects with peripheral blood eosinophilia, confirming the observations by Annema and coworkers (16) and Ulrik (17) of higher counts in both symptomatic and asymptomatic hyperresponsive men and young adults.

This study does not elucidate why peripheral blood eosinophils are related to BHR. Eosinophils play an important role in the inflammatory process of the airways of asthmatics (6). Activated eosinophils in the bronchial wall of asthmatics release arginine-rich products such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO), causing BHR due to epithelial damage and increased permeability (8, 9). Whether increased numbers of peripheral blood eosinophils reflect an inflammatory process in the airways of asymptomatic subjects that is responsible for BHR is unknown. So far, there are only two studies available showing no difference in the number of eosinophils in sputum (30) or airway wall (31) from asymptomatic children and adults with and without BHR. Therefore, it may well be that another common underlying mechanism is responsible for both peripheral blood eosinophilia and BHR. Increased numbers of eosinophils in peripheral blood may suggest a signal to the bone marrow (e.g., interleukin-5 released by T lymphocytes in the blood) that recruits eosinophils to the circulation and allows recruitment of eosinophils into the airways wall via endothelial adhesion and chemotactic factors, yet only after certain stimuli. Another possibility is that eosinophils are residing in peripheral airways, as has been observed in asthma (32). Eosinophils in either the peripheral or the central airways might create a local basic environment that easily permits the eventual development and persistence of symptoms. This possibility is supported by our finding that peripheral blood eosinophilia in asymptomatic subjects is predictive of BHR, particularly in those subjects who have been intermittently symptomatic in the past. Moreover, recent longitudinal analyses of the Vlagtwedde–Vlaardingen Study by our group have shown that BHR in asymptomatic subjects is a risk factor for subsequent development of respiratory symptoms (2).

Previous studies have shown that subjects with BHR are more likely than those without BHR to have positive skin tests (11, 12). In our study, skin test positivity was associated with BHR, yet only in symptomatic subjects: symptomatic skin test–positive subjects had an almost sixfold higher risk of BHR than symptomatic skin test–negative subjects. Earlier studies demonstrated that allergen exposure results in BHR and respiratory symptoms in atopic asthmatics (21, 33). Thus allergen exposure might account for respiratory symptoms in skin test–positive individuals.

In contrast to the findings of most other studies (27–29), we observed no relationship between high serum total IgE levels and BHR in our middle-aged population. Higher serum total IgE levels are known to be associated with smoking, male gender, and younger age. Smoking is associated with both increased serum total IgE levels and BHR. Without adjustment for smoking, a high serum total IgE level was also not associated with BHR in our study. Thus, smoking is unlikely to explain differences between our study and others. Serum total IgE levels in females are reported to be 26% lower than those in males (34). Use of separate definitions for high IgE levels in males and females (as the highest quartiles, e.g., $\geq$ 57 kU/L for females and $\geq$ 80 kU/L for males) did not change our results (OR = 1.30, 95% CI = 0.88 to 1.93). Compared with the two previously reported studies on the independent relationships of the three measures of atopy to BHR (28, 29), our study concerned not only males but both males and females, and our population was older than that studied by Boezen and coworkers (29). Because serum total IgE levels in females are lower than those in males and because IgE levels decline with age (34), the range of levels in our population is probably smaller than that in previous studies and perhaps too small to detect an association with BHR.

The definition of BHR used in our study was more stringent than the commonly used definition i.e., a PC_{20} of $\leq$ 8 mg/ml (2 min of inhalation), which is comparable to a PC_{10} of $\leq$ 16 mg/ml (30 s of inhalation) (35). The threshold value was based on a 10% decrease in FEV_1 or IVC. Of all subjects considered to have BHR, more than 30% were so categorized solely on the basis of a decrease in IVC. To reduce the likelihood of misclassification, we lowered the threshold value. If some subjects were nevertheless misclassified as having BHR, the observed relationships would have been weakened. However, repetition of the analyses with a definition of BHR as only a 10% decrease in FEV_1 (excluding subjects with a PC_{10}[IVC] of $\leq$ 8 mg/ml histamine) did not change the results.

Finally, we found a higher risk of BHR in females than males only among asymptomatic individuals. The difference in the relationship between gender and BHR for symptomatic and asymptomatic subjects disappeared after the level of FEV_1 was taken into account. This confirms other study results (36, 37). Leynaert and colleagues (36) suggested adjusting for FEV_1 as the percentage predicted in order to control for potential bias when comparing BHR in males and females with similar FEV_1 values. These investigators observed a higher risk of BHR in females when FEV_1 percentage predicted was taken into account. Our results confirmed these findings, indicating that females are more likely to have BHR than males. However, it is difficult to separate the effects of smoking and gender in these data because more males than females smoke. Although we adjusted in our analyses for smoking habits and gender, we cannot determine whether the different relation to BHR by symptom status is driven by smoking or gender.

In conclusion, the results of our study in a general adult population show that peripheral blood eosinophilia is associated with BHR in symptomatic as well as in asymptomatic subjects, whereas skin test positivity is strongly associated with BHR in symptomatic subjects only. Peripheral blood eosinophilia in subjects with BHR may facilitate recruitment and activation of eosinophils in the airway wall, giving rise to inflammatory changes resulting in intermittent respiratory symptoms upon inhalation of appropriate stimuli. This requires further study.

***Acknowledgment*:** The authors thank the participants from Vlagtwedde and Vlaardingen for their cooperation.

References

1. Jansen, D. F., W. Timens, J. Kraan, B. Rijcken, and D. S. Postma. 1997. (A)symptomatic bronchial hyperresponsiveness and asthma. *Respir. Med.* 91:121–134.
2. Xu, X., B. Rijcken, J. P. Schouten, and S. T. Weiss. 1997. Airways responsiveness and development and remission of chronic respiratory symptoms in adults. *Lancet* 350:1431–1434.
3. Hopp, R. J., R. G. Townley, R. E. Biven, A. K. Bewtra, and N. M. Nair. 1990. The presence of airway reactivity before the development of asthma. *Am. Rev. Respir. Dis.* 141:2–8.
4. Jones, A. 1994. Asymptomatic bronchial hyperreactivity and the development of asthma and other respiratory tract illnesses in children. *Thorax* 49:757–761.
5. Laprise, C., and L. P. Boulet. 1997. Asymptomatic airway hyperrespon-

siveness: a three-year follow-up. *Am. J. Respir. Crit. Care Med.* 156: 403–409.
6. Bochner, B. S., B. J. Undem, and L. M. Lichtenstein. 1994. Immunological aspects of allergic asthma. *Annu. Rev. Immunol.* 12:295–335.
7. Smith, C. H., J. N. W. N. Barker, and T. H. Lee. 1993. Adhesion molecules in allergic inflammation. *Am. Rev. Respir. Dis.* 148:S75–S78.
8. Gleich, G. J., N. A. Flavahan, T. Fujisawa, and P. M. Vanhoutte. 1988. The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J. Allergy Clin. Immunol.* 81:776–781.
9. Wardlaw, A. J., S. Dunnette, G. J. Gleich, J. V. Collins, and A. B. Kay. 1988. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma: relationship to bronchial hyperreactivity. *Am. Rev. Respir. Dis.* 137:62–69.
10. O'Connor, G. T., D. Sparrow, and S. T. Weiss. 1989. The role of allergy and nonspecific airway hyperresponsiveness in the pathogenesis of chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* 140:225–252.
11. Trigg, C. J., J. B. Bennett, M. Tooley, B. Sibbald, M. F. D'Souza, and R. J. Davies. 1990. A general practice based survey of bronchial hyperresponsiveness and its relation to symptoms, sex, age, atopy, and smoking. *Thorax* 45:866–872.
12. Britton, J., I. Pavord, K. Richards, A. Knox, A. Wisniewski, I. Wahedna, W. Kinnear, A. E. Tattersfield, and S. T. Weiss. 1994. Factors influencing the occurrence of airway hyperreactivity in the general population: the importance of atopy and airway calibre. *Eur. Respir. J.* 7:881–887.
13. Omenaas, E., P. Bakke, G. E. Eide, S. Elsayed, and A. Gulsvik. 1996. Serum house dust mite antibodies: predictor of increased bronchial responsiveness in adults of a community. *Eur. Respir. J.* 9:919–925.
14. Sunyer, J., and A. Munoz. 1996. Concentrations of methacholine for bronchial responsiveness according to symptoms, smoking, and immunoglobulin E in a population-based study in Spain. *Am. J. Respir. Crit. Care Med.* 153:1273–1279.
15. Taylor, K. J., and A. R. Luksza. 1987. Peripheral blood eosinophil counts and bronchial responsiveness. *Thorax* 42:452–456.
16. Annema, J. T., D. Sparrow, G. T. O'Connor, B. Rijcken, G. H. Koëter, D. S. Postma, and S. T. Weiss. 1995. Chronic respiratory symptoms and airway responsiveness to methacholine are associated with eosinophilia in older men: the Normative Aging Study. *Eur. Respir. J.* 8: 62–69.
17. Ulrik, C. S. 1996. Factors associated with increased bronchial responsiveness in adolescents and young adults: the importance of adjustment for prechallenge FEV_1. *J. Allergy Clin. Immunol.* 97:761–767.
18. van der Lende, R. 1969. Epidemiology of chronic non-specific lung disease (chronic bronchitis). Thesis. Royal van Gorcum, Assen.
19. van der Lende, R., T. J. Kok, R. Peset, P. H. Quanjer, J. P. Schouten, and N. G. M. Orie. 1981. Decreases in VC and FEV_1 with time: indicators for effects of smoking and air pollution. *Bull. Eur. Physiopathol. Respir.* 17:775–792.
20. van der Lende, R., and N. G. M. Orie. 1972. The MRC-ECCS questionnaire on respiratory symptoms (use in epidemiology). *Scand. J. Respir. Dis.* 53:218–226.
21. de Vries, K., J. T. Goei, H. Booy-Noord, and N. G. M. Orie. 1962. Changes during 24 hours in the lung function and histamine hyperreactivity of the bronchial tree in asthmatic and bronchitic patients. *Int. Arch. Allergy* 20:93–101.
22. Eiser, N. M., K. F. Kerrebijn, and P. H. Quanjer. 1983. Guidelines for standardization of bronchial challenges with (nonspecific) bronchoconstricting agents. *Bull. Eur. Physiopathol. Respir.* 19:495–514.
23. Mensinga, T. T., J. P. Schouten, B. Rijcken, S. T. Weiss, F. E. Speizer, and R. van der Lende. 1990. The relationship of eosinophilia and positive skin test reactivity to respiratory symptom prevalence in a community-based population study. *J. Allergy Clin. Immunol.* 86:99–107.
24. Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods, 7th ed. Iowa State University Press, Ames, Iowa.
25. Mensinga, T. T., S. T. Weiss, J. G. R. de Monchy, J. P. Schouten, B. Rijcken, and H. F. Kauffman. 1993. Smoking modifies the relationship of markers of allergy to eosinophil count in a community-based population study. Thesis, University of Groningen, Groningen, The Netherlands, 39–58.
26. Burrows, B., F. D. Martinez, M. Halonen, R. A. Barbee, and M. G. Cline. 1989. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N. Engl. J. Med.* 320:271–277.
27. Peat, J. K., B. G. Toelle, J. Dermand, R. Van den Berg, W. J. Britton, and A. J. Woolcock. 1996. Serum IgE levels, atopy, and asthma in young adults: results from a longitudinal cohort study. *Allergy* 51:804–810.
28. O'Connor, G. T., D. Sparrow, M. R. Segal, and S. T. Weiss. 1989. Smoking, atopy, and methacholine airway responsiveness among middle-aged and elderly men. *Am. Rev. Respir. Dis.* 140:1520–1526.
29. Boezen, H. M., D. S. Postma, J. P. Schouten, H. A. M. Kerstjens, and B. Rijcken. 1996. PEF variability, bronchial responsiveness and their relation to allergy markers in a random population (20–70 yr). *Am. J. Respir. Crit. Care Med.* 154:30–35.
30. Pin, I., S. Radford, R. Kolendowicz, B. Jennings, J. A. Denburg, F. E. Hargreave, and J. Dolovich. 1993. Airway inflammation in symptomatic and asymptomatic children with methacholine hyperresponsiveness. *Eur. Respir. J.* 6:1249–1256.
31. Power, C., S. Sreenan, B. Hurson, C. Burke, and L. W. Poulter. 1993. Distribution of immunocompetent cells in the bronchial wall of clinically healthy subjects showing bronchial hyperresponsiveness. *Thorax* 48:1125–1129.
32. Kraft, M., R. Djukanovic, S. Wilson, S. T. Holgate, and R. J. Martin. 1996. Alveolar tissue inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 154:1505–1510.
33. de Monchy, J. G. R., H. F. Kauffman, P. Venge, G. H. Koeter, H. M. Jansen, H. J. Sluiter, and K. de Vries. 1985. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.* 131:373–376.
34. Burney, P., E. Malmberg, S. Chinn, D. Jarvis, C. Luczynska, and E. Lai. 1997. The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J. Allergy Clin. Immunol.* 99: 314–322.
35. Rijcken, B., J. P. Schouten, S. T. Weiss, A. F. Meinesz, K. de Vries, and R. van der Lende. 1989. The distribution of bronchial responsiveness to histamine in symptomatic and asymptomatic subjects: a population based analysis of various indices of responsiveness. *Am. Rev. Respir. Dis.* 140:615–623.
36. Leynaert, B., J. Bousquet, C. Henry, R. Liard, and F. Neukirch. 1997. Is bronchial hyperresponsiveness more frequent in women than in men? *Am. J. Respir. Crit. Care Med.* 156:1413–1420.
37. Kanner, R. E., J. E. Connett, M. D. Altose, A. S. Buist, W. W. Lee, D. P. Tashkin, and R. A. Wise. 1994. Gender difference in airway hyperresponsiveness in smokers with mild COPD: the Lung Health Study. *Am. J. Respir. Crit. Care Med.* 150:956–961.

American Journal of Pathology, Vol. 163, No. 5, November 2003

Interleukin-12-Independent Down-Modulation of Cockroach Antigen-Induced Asthma in Mice by Intranasal Exposure to Bacterial Lipopolysaccharide

Steven K. Lundy, Aaron A. Berlin, and Nicholas W. Lukacs

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

Several studies have shown that exposure to bacterial lipopolysaccharide (LPS) can either prevent or inhibit asthma in humans and laboratory rodents. Much emphasis has been placed on the role of cytokines and chemokines in the establishment and maintenance of allergic airway disease. Therefore, it is of interest to study the role of LPS in affecting airway pathology and lung cytokine and chemokine responses in the maintenance phase of asthma. Increasing doses of LPS were administered into the airways of mice presensitized with cockroach allergen (CRAg), then allergic airway disease parameters were assessed after CRAg challenge. Airway hyperresponsiveness after antigen challenge decreased at the highest dose of LPS tested, which was accompanied by a decrease in airway and lung eosinophils. However, a dramatic increase in lung inflammation because of neutrophil influx was observed. Measurement of cytokines in lungs of LPS-treated, CRAg-sensitized mice indicated that interleukin (IL)-12 levels were increased by LPS treatment in a dose-dependent manner, as were levels of several inflammatory chemokines. In contrast, levels of IL-4, IL-13, IL-5, and IL-10 were reduced in whole lung homogenates only of high-dose LPS-treated mice. Intranasal administration of neutralizing anti-IL-12 at the time of high-dose LPS challenge reduced lung IL-12, interferon-γ, CXCL9, and CXCL10 but did not affect levels of the other chemokines or Th2-type cytokines, and did not restore AHR. These findings suggest that the amelioration of airway hyperresponsiveness observed in LPS-treated, CRAg-sensitized mice is coincident with an immune deviation of the lung inflammatory response, independent of IL-12. ***(Am J Pathol 2003, 163:1961–1968)***

The incidence and severity of asthma has risen dramatically throughout the past few decades, with the greatest increases being evident in the well-developed nations of North America and Europe.[1] The disease has been primarily associated with allergens derived from plant pollens or household pests such as dust mites and cockroaches, which are fairly evenly distributed throughout the world. Inhalation of aerosolized allergen leads to inflammation of the major airways of the lung, which is mediated by cytokines and chemokines of the innate and Th2-type adaptive immune response.[2,3] Asthma is characterized by production of allergen-specific IgE, mast cell activation, influx of eosinophils into the lung and airway hyperproduction of mucus, and increased sensitivity of airway smooth muscle to neurotransmitters leading to bronchoconstriction and decreased air capacity.

Increased sanitation and antibiotic usage and decreased environmental exposure to immunogenic organisms including bacteria have been linked to increasing incidence and severity of asthma in developed countries leading to the hygiene hypothesis.[4–7] A recent epidemiological report clearly demonstrated an inverse correlation between several markers of allergy and atopy of a large cohort of children and the amount of endotoxin [lipopolysaccharide (LPS)] found in their bedding material.[8] This study is in agreement with several other studies showing decreased incidence of asthma in children raised in a farming environment and associations based on household size and birth order.[9–11] However, a significant amount of data has shown that exposure to endotoxin in the workplace or as a component of cigarette smoke leads to exacerbation of pre-existing asthma.[12–15] Thus, it remains unclear what role exposure to endotoxin plays in modulation of human allergic airway disease.

Studies in animal models of asthma using ovalbumin (OVA) as the allergen have also been contradictory, with nearly as many investigators reporting that endotoxin inhibits asthma-associated airway inflammation as those reporting enhancement.[16–21] Eisenbarth and colleagues[22] have shown that the allergic response to inhaled OVA is dependent on activation through toll-like receptor 4 by low-dose contamination with LPS, but that a higher dose of LPS inhibited development of asthma. The difference in response was associated with drastic changes in leukocyte migration, immunoglobulin isotype switching, and cytokine expression, such that the highly

Support by the National Institutes of Health (grant AI-36302).

Accepted for publication August 4, 2003.

Address reprint requests to Nicholas W. Lukacs, Department of Pathology, University of Michigan Medical School, 5214 Medical Sciences I, 1301 Catherine St., Ann Arbor, MI 48109. E-mail: nwlukacs@umich.edu.

Th2-type response elicited by low-dose LPS was reversed to a predominant Th1-type pattern at the higher dose. These data suggested that there could be a therapeutic dose of endotoxin that would be effective at treating asthma.

In the current study, we hypothesized that intranasal endotoxin exposure would cause immune deviation and reverse asthmatic disease in mice presensitized to cockroach antigens (CRAg). Increasing doses of intranasal LPS were administered to CRAg-sensitized mice before a final intratracheal challenge with CRAg and measurement of asthma parameters. The lowest dose of LPS tested appeared to decrease airway hyperreactivity (AHR). As the dose of LPS increased, AHR was progressively reduced and the pattern of cytokine expression changed from a high-Th2/low Th1-type to a high-Th2/high-Th1-type, then to a low-Th2-type/high-Th1-type response. Alterations in lung chemokine expression, a dramatic influx of neutrophils and $CD4^+$ T lymphocytes, and a decrease in lung eosinophilia were also observed in LPS-treated mice. Neutralization of interleukin (IL)-12 by intranasal anti-murine IL-12 treatment caused a reduction in Th1-cytokine expression but did not lead to increased Th2 cytokine expression or reconstitution of AHR.

Materials and Methods

Animals and Reagents

Female CBA/J mice (8 to 10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in covered boxes (five mice/box) in the Unit for Laboratory Animal Medicine (ULAM) facility of the University of Michigan. Clinical skin-test grade cockroach antigen extract was purchased from Hollister-Stier (Spokane, WA), small molecular weight components were removed by centrifugation through an Amicon 3000 column, and batches were tested for the absence of endotoxin by limulus amoebocyte assay (detection limit 6 pg/ml; BioWhittaker, Walkersville, MD) before use. Incomplete Freund's adjuvant and bacterial LPS (*Escherichia coli* serotype 0111:B4, lot no. 70K4108) were purchased from Sigma (St. Louis, MO). LPS was diluted to 1 mg/ml in sterile saline and sonicated for 1 hour before aliquoting and freezing. A fresh aliquot was thawed and vigorously shaken before the start of each experiment. Neutralizing anti-murine IL-12 antibodies were collected from polyclonal anti-serum of rabbits immunized and rechallenged with recombinant murine IL-12. Normal rabbit serum was collected and used to control for the presence of rabbit immunoglobulins. The rabbit antibodies were purified over a protein A column and diluted to a total protein concentration of 10 mg/ml in sterile saline before use.

Antigen Sensitization and Challenge

See Figure 1 for a schematic timeline of the sensitization protocol. Cockroach antigen (20,000 PNU/ml) was mixed at a ratio of 1:1 with incomplete Freund's adjuvant and 0.1 ml of emulsion was injected both intraperitoneally and subcutaneously at the nape of the neck. Fourteen days later, mice were anesthetized with ketamine and xylazine, then 15 μl of undiluted CRAg was injected intranasally. A 15-μl dose of the indicated amount of LPS or sterile saline diluent was given intranasally to anesthetized mice on days 15, 17, 19, and 21 after CRAg sensitization. Antibody-treated mice received an intranasal dose of anti-murine IL-12 or normal rabbit serum (10 μl) at the same time as LPS administration. Immediately after the final dose of LPS, the trachea was exposed by incision, 40 μl of undiluted CRAg was injected down the airway, and the wound was closed with wound clips.

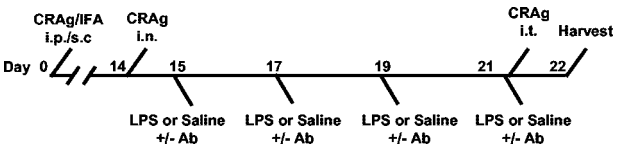


Figure 1. Female CBA/J mice were sensitized on day 0 by intraperitoneal and subcutaneous immunization with CRAg emulsified in IFA. An intranasal challenge with undiluted CRAg was given on day 14, followed by four alternate-day intranasal exposures to LPS or saline starting on day 15. Some mice received intranasal treatments with normal rabbit serum or neutralizing anti-IL-12 antibodies at the time of LPS exposure. Mice received a final intratracheal challenge with undiluted CRAg on day 21, followed by assessment of AHR and collection of tissue samples 18 to 20 hours after intratracheal challenge. The CRAg used in these studies tested negative (<6 pg/ml) for LPS by limulus amoebocyte assay.

Measurement of AHR and Harvest of Tissues

Mice were anesthetized with sodium pentobarbital (3.3 mg/mouse) 18 to 20 hours after intratracheal CRAg challenge, trachea were exposed, an airway tube was inserted, and mice were connected directly to a plethysmograph (Buxco, Troy, NY). A baseline of airway resistance was established for each mouse, then methacholine (6.25 μg in 0.1 ml) was administered intravenously and airway resistance was measured again. Peak airway resistance after methacholine challenge was divided by baseline to determine the fold increase in AHR. Bronchoalveolar cells and fluid were collected by lavage with 1.0 ml of sterile saline. Blood was obtained by orbital bleed, followed by sacrifice and removal of the lungs.

Flow Cytometry

Total right lobe $CD4^+$ T cells were determined by collagenase dispersion of cells from the whole right lobe of each mouse, followed by a total cell count with trypan blue. Dispersed cells were stained with FcBlock (BD Biosciences, San Diego, CA), followed by staining with phycoerythrin-conjugated anti-murine CD4 (BD Biosciences), and analyzed on a Beckman Coulter Epics XL flow cytometer (Brea, CA). The total number of cells in the right lobe was multiplied by the percentage of $CD4^+$ cells to determine total $CD4^+$ T-cell content.

Histology

Lungs were removed after analysis of AHR and inflated with 10% neutral buffered formalin before paraffin embedding. Tissue morphology and lung eosinophil content

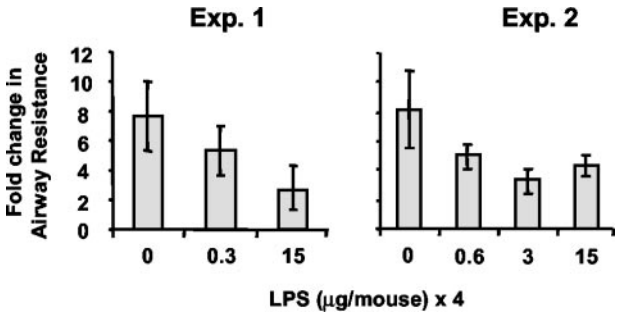


Figure 2. Anesthetized mice were analyzed for airway hyperresponsiveness before and after methacholine challenge at 18 to 20 hours after intratracheal challenge. Data are the fold change in airway resistance after methacholine administration ± SE for three to five mice from two separate experiments. The cumulative data from four experiments using the 15-μg/ml dose of intranasal LPS (n = 11 mice) indicated a statistically significant decrease in AHR ($P < 0.05$) compared to saline control mice (n = 16 mice).

were assessed on hematoxylin and eosin-stained sections from paraffin-embedded, right lobes. Lung neutrophil, eosinophil, monocyte/macrophage, and lymphocyte content was determined from Diffquick (Dade Behring, Newark, DE)-stained cytospins of the cells dispersed for flow cytometry. Bronchoalveolar lavage (BAL) cells were centrifuged and resuspended in 1.0 ml of ACK red blood cell lysis buffer for 3 minutes before immobilizing 0.2 ml of the cell suspension on a microscope slide by cytospin and staining with Diffquick. Cell differentials were counted using an Olympus BX40 microscope at ×1000 magnification and photographs were taken with a Spot RT color camera (Diagnostic Instruments, Sterling Heights, MI).

Measurement of Cytokines

The left lobe of each lung was snap-frozen and homogenized in 1.0 ml of 0.1%Triton X-100:phosphate-buffered saline containing protease inhibitors before analysis of cytokine content by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) following manufacturer's instructions. Total protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA). The cytokine content of each lung was normalized to total protein and then mean and SE were determined within each treatment group.

Statistical Analysis

Multiple pairwise comparisons were performed using the analysis of variance portion of the Prism software. Individual pairwise comparisons were performed using Student's t-test.

Results

Bronchoconstriction is a well-established marker of asthma and is tested for clinically by measurement of airway capacity after administration of methacholine. Mice sensitized with CRAg responded to methacholine with a sevenfold to eightfold increase in airway hyperresponsiveness (AHR) compared to baseline airway resistance before methacholine challenge (Figure 2). Repeated intranasal exposure to increasing doses of LPS during the course of CRAg sensitization led to an apparent dose-dependent decrease in airway hyperresponsiveness.

Serum levels of IgE were higher in CRAg-sensitized mice than in unsensitized animals but treatment with LPS did not significantly affect systemic IgE levels (Table 1). The lungs of LPS-treated mice were highly inflamed (Figure 3) with an increase in the presence of total leukocytes and $CD4^+$ T cells within the lung tissue and a dramatic increase in the presence of neutrophils in the BAL fluid (Table 1). The number of eosinophils in the BAL and surrounding the bronchi was sharply reduced with the highest dose of LPS, but was not significantly affected at the lower doses (Table 1).

Treatment with LPS led to a dose-dependent increase in IL-12 production in the mouse lung 18 hours after airway exposure (Figure 4), however, neither interferon (IFN)-γ nor tumor necrosis factor-α levels in the lung were increased by intranasal treatment with LPS (data not shown). Levels of the Th2-associated cytokines, IL-4, IL-13, IL-5, and IL-10 were not decreased by the low-

Table 1. Increased Neutrophilia and Decreased Eosinophilia in LPS-Expressed CRAg-Sensitized Mice

			Lung (Rt. lobe)			Bronchoalveolar lavage			
Group	n	Total serum IgE (μg/ml)*	Leukocytes ($\times 10^6$)	$CD4^+$ ($\times 10^5$)	Peribronchial eosinophils (cells/20 HPF)	Total leukocytes (cells/HPF)	PMN (%)	Eosinophils (%)	Lymphs (%)
Exp. 1									
No LPS	4	5.4 ± 1.4†	6.7 ± 0.7	4.9 ± 0.6	N.D.‡	15.5 ± 2.5	48.8 ± 4.8	4.3 ± 1.5	2.4 ± 0.4
LPS, 0.3 μg	5	6.6 ± 1.9	9.0 ± 0.8§	6.2 ± 0.7	N.D.	21.1 ± 2.0§	70.2 ± 6.0§	6.3 ± 2.4	1.4 ± 0.6
LPS, 15 μg	3	6.4 ± 1.7	20.9 ± 1.0§	10.6 ± 0.5§	N.D.	47.1 ± 3.1§	81.2 ± 0.4§	0.0 ± 0.0§	0.9 ± 0.1§
Exp. 2									
No LPS	4	4.8 ± 1.2	N.D.	N.D.	139 ± 59	14.3 ± 2.4	44.9 ± 10.6	9.2 ± 5.9	2.2 ± 0.3
LPS, 0.6 μg	4	3.6 ± 1.2	N.D.	N.D.	77 ± 20	27.1 ± 2.8§	82.3 ± 2.4§	1.2 ± 0.4§	0.9 ± 0.3§
LPS, 3 μg	4	4.5 ± 2.3	N.D.	N.D.	62 ± 24	25.8 ± 2.3§	82.1 ± 3.6§	3.1 ± 1.4	1.8 ± 0.5
LPS, 15 μg	3	4.2 ± 1.5	N.D.	N.D.	18 ± 6§	62.3 ± 5.4§	93.1 ± 2.8§	0.1 ± 0.1§	0.8 ± 0.5§

*Serum IgE level in non-CRAg-sensitized mice was 1.0 ± 0.4 μg/ml of serum.
†Data are mean ± standard error.
‡N.D. = not determined.
§Statistically significant difference ($P < 0.05$) from no LPS control.

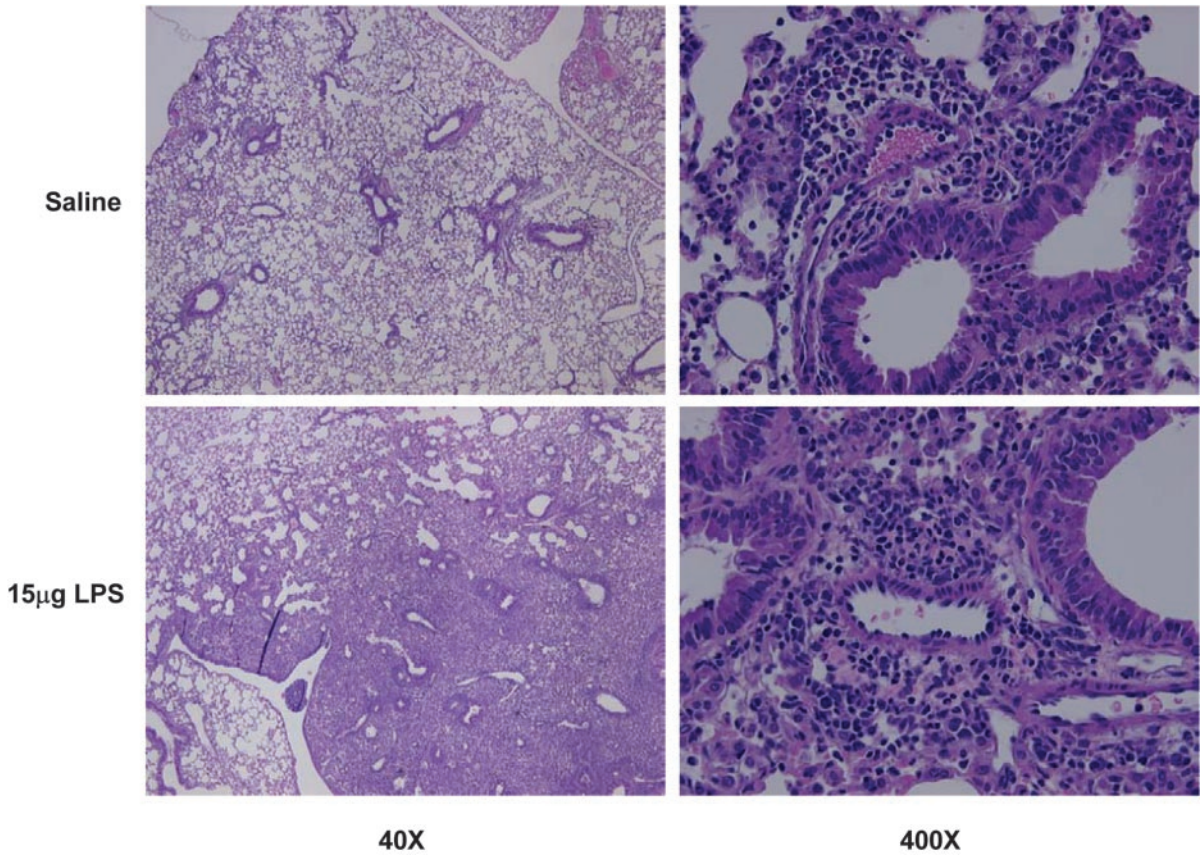


Figure 3. Lungs were removed 18 to 20 hours after intratracheal CRAg challenge and paraffin-embedded. Sections (5 μm) were stained with H&E and photographed at the indicated magnification. Lungs of CRAg-sensitized mice exposed to intranasal saline had modest levels of inflammation with evidence of peribronchial eosinophilia. Lungs of CRAg-sensitized mice given intranasal LPS (15 μg $\times$ 4 doses) had large areas of severe inflammation and airways surrounded by cellular infiltrates consisting primarily of polymorphonuclear neutrophils and monocytes.

dose LPS treatment that caused progressive elevation of IL-12, but the levels of all of these Th2 cytokines were diminished at the highest dose of LPS (Figure 4).

Leukocyte recruitment to the lung during inflammation is partially dependent on the release of chemokines from T cells, leukocytes, and structural cells. Treatment with LPS caused a dose-dependent increase in the level of CXCL8/MIP-2, CCL2/JE, and CCL20/MIP-3α, that have been associated with the innate inflammatory response (Figure 5). Similarly, increases in Th1-associated chemokines, CCL3/MIP-1α, CCL5/RANTES, CXCL9/MIG, and CXCL10/IP-10 were noted after airway exposure to increasing doses of LPS. Although lung levels of the Th2-associated chemokines, CCL11/Eotaxin, CCL17/TARC, and CCL22/MDC were not significantly reduced by LPS exposure (data not shown), CCL1/TCA-3 was reduced by high-dose LPS treatment, similar to the findings for the Th2-associated cytokines (Figure 5).

A similar increase of innate and Th1-associated cytokines and chemokines was observed in the BAL fluid of intranasal LPS-exposed mice (Figure 6). Unlike the sustained levels of IL-10 observed in the whole lung after intermediate doses of LPS, the levels of IL-10 in BAL fluid dropped progressively in a LPS dose-dependent manner.

The dose-dependent increase in Th1-associated chemokines prompted interest in the role of IL-12 to the modulation of AHR and inflammation after LPS exposure. Neutralization of airway IL-12 by intranasal exposure at the time of each high-dose LPS administration did not affect the down-regulation of AHR (Figure 7) despite decreasing BAL levels of IL-12, CXCL9, and CXCL10. Although whole lung levels of IL-12, IFN-γ, CXCL9, and CXCL10 levels were significantly reduced after anti-IL-12 treatment (Figure 8), no reciprocal increase in the Th2-associated cytokines IL-4, IL-5, IL-13 (Figure 8); or the Th2-associated chemokines CCL1, CCL11, CCL17, or CCL22 (data not shown) was detected in the lungs of antibody-treated mice. Further, the chemokines CCL2, CCL3, CCL5, and CXCL8, that were all induced by LPS exposure, were not significantly affected by airway installation of anti-IL-12 antibody.

Intranasal IL-12 neutralization at the time of LPS exposure did not lead to a significant change in total serum IgE. (Table 2) However the number of leukocytes in the BAL fluid was significantly reduced and an apparent increase in peribronchial eosinophilia was also observed after IL-12 neutralization, but did not reach statistical significance (Table 2).

Discussion

In the current study, bacterial LPS was shown to deviate the inflammatory response in a cockroach allergen

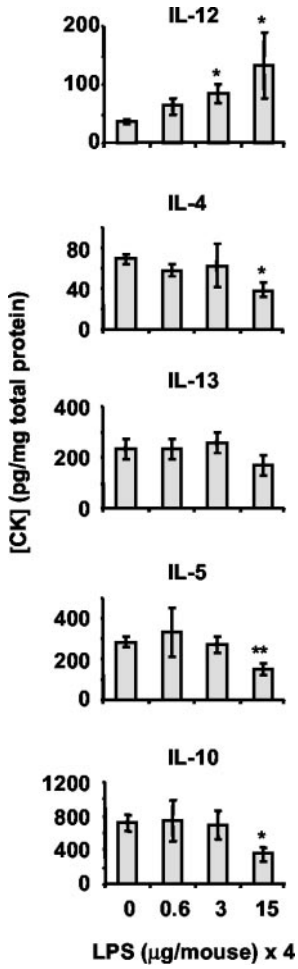


Figure 4. Lungs of CRAg-sensitized, untreated, and LPS exposed mice were removed after analysis of AHR and snap-frozen. Whole lung homogenates were analyzed by sandwich enzyme-linked immunosorbent assay for the cytokines listed. Additional analysis was done for IFN-γ, tumor necrosis factor-α, and transforming growth factor-β but no significant differences were detected in any experiment (not shown). Data are the mean cytokine concentration ± SE for three to five mice/group in this representative experiment of four performed. The increase in IL-12 was significant ($P < 0.05$) at the 3 μg dose compared to the untreated control. Decreased IL-4, IL-5, and IL-10 showed significance at the 15-μg dose ($P < 0.05$, 0.02, and 0.05, respectively). Although the decrease in IL-13 did not reach statistical significance in this experiment, it was consistently reduced in all experiments at the 15-μg dose, and did reach significance in several experiments.

(CRAg)-induced model of asthma. Repeated intranasal exposure to LPS led to a dose-dependent decrease in AHR after methacholine administration. This finding is in agreement with several epidemiological studies that have shown an inverse relationship between human asthma and environmental exposure to LPS.[8–10,23] Several studies in animal models have shown a similar decrease in AHR when LPS exposure was combined with airway allergen challenge. Cochran and colleagues[16] demonstrated that a single intranasal dose of 1 μg of LPS given to young mice decreased AHR when the mice were subsequently challenged with OVA. In a study more similar to our current protocol, Tulic and colleagues[19] demonstrated that LPS exposure of rats that were presensitized to OVA led to a LPS dose-dependent decrease in sensitivity to methacholine.

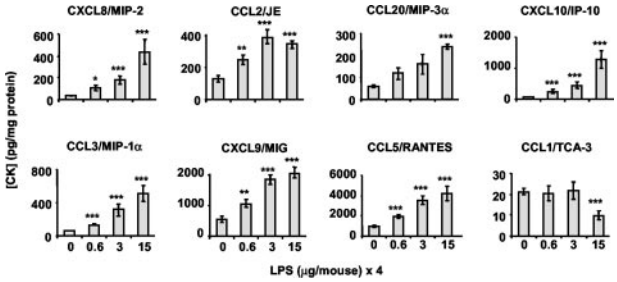


Figure 5. Whole lung homogenates were analyzed for chemokine expression by sandwich enzyme-linked immunosorbent assay. Data shown are mean chemokine concentration ± SE for three to five mice/group in this representative experiment of four performed. Levels of CXCL8, CCL2, CXCL10, CXCL9, CCL3, and CCL5 were significantly increased after exposure to the lowest dose of LPS compared to untreated controls ($P < 0.05$, 0.02, 0.01, 0.02, 0.01, and 0.01, respectively). Increased CCL20 reached significance at the 15-μg dose of LPS ($P < 0.001$). Decreased CCL1 ($P < 0.01$) was detected at the 15-μg dose of LPS. Whole lung levels of CCL22/MDC were unchanged by LPS exposure, but CCL11/eotaxin and CCL17/TARC were nonsignificantly reduced at the 15-μg dose of LPS in all experiments (not shown).

A well-established mechanism of induction of AHR in asthma is the release of leukotrienes from mast cell granules after crosslinkage of antigen-specific IgE on the mast cell surface. The current study showed that serum IgE levels were not significantly affected by intranasal exposure to LPS. A recent study by Gerhold and colleagues[17] showed that systemic exposure to LPS before OVA sensitization led to prevention of OVA-specific IgE production, however, that study also indicated that intranasal LPS treatment did not affect total or antigen-specific IgE production, in agreement with the current findings. However, in contrast to our current findings, Gerhold and colleagues[17] did not detect any change in

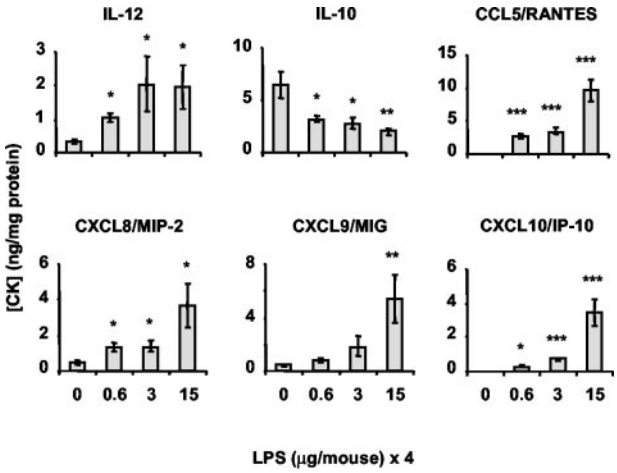


Figure 6. BAL was performed by injection of 1 ml of sterile saline into a tracheal tube followed by aspiration. Cells were removed by centrifugation and the supernatant was frozen until tested by enzyme-linked immunosorbent assay for the indicated cytokines and chemokines. Total protein content in BAL was determined with the Bradford reagent and cytokine levels were normalized to protein content for each sample. Data are mean concentration ± SE for three to five mice/group in this representative experiment of four performed. A significant increase in total BAL protein ($P < 0.02$) was detected with high-dose LPS exposure. IL-10 levels were reduced ($P < 0.05$) and IL-12, CCL5, CXCL8, and CXCL10 levels were increased by the lowest dose of LPS ($P < 0.01$, 0.001, 0.001 and 0.02, respectively). The increase in CXCL9 was significant ($P < 0.02$) after high-dose LPS exposure. IL-4, IL-5, IL-13, and IFN-γ were below detectable levels in the BAL fluid of these mice.

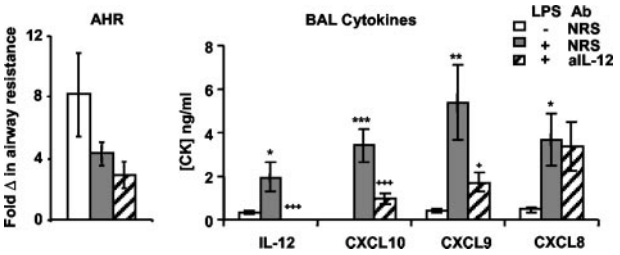


Figure 7. AHR and BAL cytokine levels were tested as described in Figures 1 and 5 after intranasal exposure of CRAg-challenged mice (18 hours) with sterile saline/normal rabbit serum (**white bars**), high-dose LPS/normal rabbit serum (**gray bars**), or high-dose LPS/anti-IL-12 (**striped bars**). Data are mean ± SE for five mice/group in this representative experiment of two performed. AHR was unchanged by anti-IL-12 treatment compared with mice receiving high-dose LPS alone. Reductions in BAL IL-12, CXCL10 ($P < 0.01$), and CXCL9 ($P < 0.05$), were evident after anti-IL-12 treatment. No statistically significant changes in other cytokines or chemokines were detected (CXCL8/MIP-2 shown as an example).

AHR after local exposure to LPS. Plausible explanations of these conflicting results include the difference in allergens and strains of mice used in these studies. Another recent study showed that low-level contamination of OVA by LPS (0.1 μg/dose) was necessary to elicit a Th2 cytokine response, OVA-specific IgE and allergic disease, whereas a high dose of LPS (100 μg/dose) was inhibitory of allergen sensitization.[22] The CRAg used in our study contained less than 6 pg/ml of LPS and was sufficient to elicit an asthmatic response in our model. The lowest dose of LPS used in the current study (0.3 μg/dose) did not exacerbate AHR induced by CRAg alone, but rather caused a slight decrease in hyperreactivity. This result suggests that CRAg does not require LPS contamination to induce asthma and that even low levels of LPS exposure are inhibitory in this model.

Airway exposure of CRAg-sensitized mice with LPS led to a dramatic influx of neutrophils into the lung parenchyma and the airway itself. An influx of $CD4^+$ Th cells was also detected in the lung but a reduced number of total lymphocytes and $CD4^+$ Th cells was detected in the BAL. At low doses of LPS, eosinophils were still present in the lung and BAL, but mice that received the highest dose of LPS displayed a marked reduction in airway and lung eosinophilia. These data, which agree with previous studies, indicate a drastic change in the inflammatory cellular response dependent on LPS.[22]

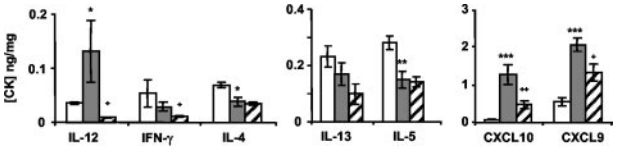


Figure 8. Lung cytokine levels were measured in anti-IL-12-treated, LPS-exposed, CRAg-sensitized mice (**striped bars**) and compared to controls that were CRAg-sensitized and received sterile saline (**white bars**) or intranasal LPS (**gray bars**) and normal rabbit serum control antibody. Significant decreases in IL-12, IFN-γ, CXCL9 ($P < 0.05$), and CXCL10 ($P < 0.02$) were observed. No changes in the Th2-associated cytokines IL-4, IL-5, or IL-13 were detected after airway neutralization of IL-12 at the time of LPS exposure. Data are mean ± SE for five mice/group in this representative experiment of two performed.

The decreased hyperreactivity was more closely associated to increased levels of IL-12 and several inflammatory and Th1-associated chemokines in the lung and BAL than to decreased levels of Th2-associated cytokines and chemokines. In fact, significant reductions of the Th2-associated cytokines were only detected at the highest dose of LPS. These findings supported the hypothesis that a diversion of the immune response from a Th2- to a Th1-type response caused the decrease in AHR. To test this hypothesis, localized neutralization of IL-12 was performed because IL-12 is a critical cytokine in the establishment of Th1-associated inflammation. Although levels of IL-12 and other Th1-associated cytokines and chemokines were reduced after anti-IL-12 instillation, the treatment had no effect on the decreased AHR resulting from LPS exposure. Surprisingly, the neutralization of lung IL-12 was not effective at restoring expression of any of the Th2-associated cytokines or chemokines tested in this study, indicating that another mechanism of Th2 suppression was in effect. Yet, it seems unlikely that the suppression of the local Th2 response alone can explain decreased AHR because decreased AHR was apparent at doses of LPS that did not suppress Th2 cytokine production in the lung.

The findings of this study shed some light on some of the currently held hypotheses of the effects of LPS on AHR.[5] Th1 immune deviation has been shown to decrease AHR, therefore, it has been suggested that LPS-induced immune deviation toward a Th1 response inhibits the Th2 response that leads to asthma.[24–30] The current findings do not support that hypothesis because neither IFN-γ nor tumor necrosis factor-α were elevated in the lungs of LPS-treated mice, and inhibition of the classical Th1 response by neutralization of IL-12 did not reverse the blockade on AHR.

Over-production of IL-10 in response to LPS has been put forward as a plausible mechanism of LPS-mediated down-regulation of airway inflammation.[5,19,31,32] Some studies have shown that local increases in the levels of IL-10 in the lung, have an anti-inflammatory effect on

Table 2. Blockade of IL-12 Decreases LPS-Induced Leukocyte Infiltration

Group	*n*	Total serum IgE (μg/ml)	Peribronchial eosinophils (cells/20 HPF)	Bronchoalveolar lavage			
				Total leukocytes (cells/HPF)	PMN (%)	Eosinophils (%)	Lymphs (%)
No LPS + NRS	7	4.1 ± 0.7*	125 ± 44	17 ± 1	48.3 ± 6.1	5.9 ± 2.6	1.7 ± 0.4
15 μg LPS + NRS	7	3.8 ± 0.4	40 ± 12	70 ± 4†	86.8 ± 5.6†	0.4 ± 0.2	1.0 ± 0.3
15 μg LPS + aIL-12	9	5.5 ± 0.9	70 ± 13	46 ± 3†‡	79.6 ± 4.6†	1.0 ± 0.2	1.2 ± 0.4

*Data are combined mean ± standard error from two separate experiments.
†Statistically significant difference ($P < 0.05$) compared to no LPS control.
‡Statistically significant difference compared to LPS + NRS group.

airway inflammation.[28,33] In the current study, there was no evidence of IL-10 induction by LPS, and in fact, the levels were markedly reduced in the BAL at low doses of LPS and in the whole lung at higher doses. To ensure that the discrepancy between our findings regarding IL-10 and those put forward by others was not because of timing of detection, we used neutralizing antibodies to IL-10 in a similar manner as for blocking IL-12. Airway neutralization of IL-10 at the time of LPS exposure had no effect on AHR, cellular infiltration, or cytokine/chemokine production in our model (data not shown).

In addition to these more commonly held hypotheses, we had entertained a role for LPS-activated, B-1a cells as a source of regulatory cytokines or as inducers of $CD4^+$ T-cell apoptosis.[34] However, studies to test the latter hypothesis in Xid, B-1 cell-deficient mice indicated no role for B-1 cells in affecting the LPS-mediated events.

Thus, it remains unclear what is the direct effector mechanism of LPS exposure on reducing AHR. There were several chemokines elevated by LPS exposure in this study that were not neutralized by our anti-IL-12 treatment. These chemokines may have been responsible for directing cellular influx and activation changes that led to decreased AHR. Another possibility is that LPS had an effect on other mediators of airway smooth muscle cell contraction. It has been suggested that changes in nitric oxide production induced by LPS are mediators of decreased AHR.[35–37] In addition, LPS may have had direct effects on airway mast cells that led to early spontaneous degranulation before the time that AHR was tested in this study.[19] Prolonged exposure to LPS could eventually lead to desensitization of mast cells, alveolar macrophages, smooth muscle cells, or other cells involved in the asthmatic response.

AHR is one of the most severe and life-threatening symptoms of asthma, therefore, LPS or one of its derivatives would seem to be a good candidate for therapy of asthmatic patients. However, a note of caution is in order. The mice exposed to high doses of LPS in this study displayed severe inflammation in their lungs, high levels of potentially dangerous Th1-associated cytokines, and an increasing mortality rate (40% at the highest dose tested). A better approach to developing therapies based on the natural effects of LPS may be to target the specific cytokines, chemokines, cellular interactions, or other inflammatory mediators involved in the decreased airway hyperreactive response. To that end, further study of the complex nature of the airway response to LPS exposure is warranted.

References

1. Redd SC: Asthma in the United States: burden and current theories. Environ Health Perspect 2002, 110(Suppl 4):557–560
2. Lukacs NW: Role of chemokines in the pathogenesis of asthma. Nat Rev Immunol 2001, 1:108–116
3. Renauld JC: New insights into the role of cytokines in asthma. J Clin Pathol 2001, 54:577–589
4. Weiss ST: Eat dirt—the hygiene hypothesis and allergic diseases. N Engl J Med 2002, 347:930–931
5. Liu AH: Endotoxin exposure in allergy and asthma: reconciling a paradox. J Allergy Clin Immunol 2002, 109:379–392
6. Anderson WJ, Watson L: Asthma and the hygiene hypothesis. N Engl J Med 2001, 344:1643–1644
7. Wills-Karp M, Santeliz J, Karp CL: The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol 2001, 1:69–75
8. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E: Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002, 347: 869–877
9. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, Vuille JC, Wuthrich B: Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999, 29:28–34
10. Strachan DP: Family size, infection and atopy: the first decade of the "hygiene hypothesis." Thorax 2000, 55(Suppl 1):S2–S10
11. Matricardi PM, Franzinelli F, Franco A, Caprio G, Murru F, Cioffi D, Ferrigno L, Palermo A, Ciccarelli N, Rosmini F: Sibship size, birth order, and atopy in 11,371 Italian young men. J Allergy Clin Immunol 1998, 101:439–444
12. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W: Bacterial endotoxin is an active component of cigarette smoke. Chest 1999, 115:829–835
13. Reed CE, Milton DK: Endotoxin-stimulated innate immunity: a contributing factor for asthma. J Allergy Clin Immunol 2001, 108:157–166
14. Rylander R, Haglind P, Lundholm M: Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. Am Rev Respir Dis 1985, 131:209–213
15. Michel O, Duchateau J, Sergysels R: Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. J Appl Physiol 1989, 66:1059–1064
16. Cochran JR, Khan AM, Elidemir O, Xue H, Cua B, Fullmer J, Larsen GL, Colasurdo GN: Influence of lipopolysaccharide exposure on airway function and allergic responses in developing mice. Pediatr Pulmonol 2002, 34:267–277
17. Gerhold K, Blumchen K, Bock A, Seib C, Stock P, Kallinich T, Lohning M, Wahn U, Hamelmann E: Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. J Allergy Clin Immunol 2002, 110:110–116
18. Tulic MK, Wale JL, Holt PG, Sly PD: Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 2000, 22:604–612
19. Tulic MK, Holt PG, Sly PD: Modification of acute and late-phase allergic responses to ovalbumin with lipopolysaccharide. Int Arch Allergy Immunol 2002, 129:119–128
20. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, Wouters EF: Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. Am J Respir Cell Mol Biol 2002, 26:152–159
21. Wan GH, Li CS, Lin RH: Airborne endotoxin exposure and the development of airway antigen-specific allergic responses. Clin Exp Allergy 2000, 30:426–432
22. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K: Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002, 196:1645–1651
23. von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S, Waser M, Nowak D: Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy 2000, 30:1230–1234
24. Iwamoto I, Nakajima H, Endo H, Yoshida S: Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. J Exp Med 1993, 177: 573–576
25. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M: Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J Exp Med 1995, 182:1527–1536
26. Kips JC, Brusselle GJ, Joos GF, Peleman RA, Tavernier JH, Devos RR, Pauwels RA: Interleukin-12 inhibits antigen-induced airway hy-

perresponsiveness in mice. Am J Respir Crit Care Med 1996, 153: 535–539
27. Sur S, Lam J, Bouchard P, Sigounas A, Holbert D, Metzger WJ: Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. J Immunol 1996, 157:4173–4180
28. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, Bowen G, Rook G, Walker C: Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 2002, 8:625–629
29. Herz U, Gerhold K, Gruber C, Braun A, Wahn U, Renz H, Paul K: BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. J Allergy Clin Immunol 1998, 102:867–874
30. Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G: Infection of mice with Mycobacterium bovis-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. J Exp Med 1998, 187: 561–569
31. Zuany-Amorim C, Haile S, Leduc D, Dumarey C, Huerre M, Vargaftig BB, Pretolani M: Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J Clin Invest 1995, 95:2644–2651
32. van Scott MR, Justice JP, Bradfield JF, Enright E, Sigounas A, Sur S: IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. Am J Physiol 2000, 278:L667–L674
33. Stampfli MR, Cwiartka M, Gajewska BU, Alvarez D, Ritz SA, Inman MD, Xing Z, Jordana M: Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. Am J Respir Cell Mol Biol 1999, 21:586–596
34. Lundy SK, Boros DL: Fas ligand-expressing B-1a lymphocytes mediate CD4(+)-T-cell apoptosis during schistosomal infection: induction by interleukin 4 (IL-4) and IL-10. Infect Immun 2002, 70:812–819
35. Tulic MK, Knight DA, Holt PG, Sly PD: Lipopolysaccharide inhibits the late-phase response to allergen by altering nitric oxide synthase activity and interleukin-10. Am J Respir Cell Mol Biol 2001, 24:640–646
36. Schuiling M, Zuidhof AB, Bonouvrie MA, Venema N, Zaagsma J, Meurs H: Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. Br J Pharmacol 1998, 123:1450–1456
37. Rodriguez D, Keller AC, Faquim-Mauro EL, De Macedo MS, Cunha FQ, Lefort J, Vargaftig BB, Russo M: Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. J Immunol 2003, 171: 1001–1008

Microbiological and Epidemiological Surveillance Programme to Monitor the Health Effects of Foray 48B BTK Spray

Submitted to:
The Ministry of Forests
Province of British Columbia

Michael A. Noble MD FRCPC [1,3]

Peter D. Riben MD MHSc FRCPC [1,3]

Gregory J. Cook MD [2]

Departments of Pathology (Medical Microbiology) [1]and Health Care and Epidemiology [2], University of British Columbia, and University Hospital [3], Vancouver, British Columbia

September 30, 1992

Table of Contents

Table of Contents

Conclusions

- After an initial period of concern prior to the commencement of the combined spray programme, people called the government telephone hot-line with health related problems with markedly decreasing frequency.

- Complaints of asthma, or other respiratory diseases, or complaints of eye, nose or throat irritation were not more common in people living inside the spray zone, nor were they more common in people who were found to be culture positive with BTK.

- There was no evidence of an increase in people attending hospital emergency departments during the time of the spray.

- People with asthma or other respiratory diseases did not present to emergency departments more frequently during or after spraying as compared to before spraying.

- No cases of serious infection with BTK were identified in any hospitalized patients.

- Occupationally exposed ground spray workers, frequently developed symptoms of headache, nose, throat and eye irritation, dry skin and chapped lips, but no days of work loss were attributable to BTK.

- BTK was repeatedly cultured from commercially available vegetables during and after the spray. People were readily exposed to sources of BTK other than either the aerial or ground sprays.

Summary

The objective of the microbiological and epidemiological surveillance study was to observe if measurable changes in health occurred during or after the combined aerial and ground spray of Foray 48B, a pesticide that contains Bacillus thuringiensis var kurstaki (BTK). In the process of addressing this question the study has examined the records of more than 26,000 telephone calls, 1140 family practice patients, 3500 admissions to hospital emergency departments, and closely monitored 120 workers with occupational exposure to BTK spray. In addition, the study examined more than 400 bacterial cultures that had been referred in from 10 participating laboratories. In addition we examined samples of air exposed to general concentrations and occupational concentrations of BTK, and samples of food from a variety of times and sources.

The results of this multi-phased study present a wide picture of the Lower Mainland before, during and after the period of aerial and ground spray, and allows some conclusions to be drawn about the impacts of BTK spray on this large urban population.

Although over 26,000 telephone calls were made to the government "MOTH-LINE", only a small proportion related to health concerns. The ranges of complaints were consistent with the problems detected by the Physician's Office Surveillance Programme, and the Ground-spray Workers Surveillance Programme. A marked decrease in the frequency of calls was noted as the spray programme progressed.

The use of the 24 family practice units as sentinel sites provided a representative sample of individuals who attend doctor's offices across the Lower Mainland, and allowed for observing the frequency of complaints in those individuals living within and outside the spray zone. Although many patients complained about respiratory and eye symptoms, we could find no evidence to support that any of the complaints were more frequent in individuals who lived within the spray zone, or in those who had objective evidence of having been exposed to spray. While symptoms may be attributable to the spray, it is not possible to distinguish these from the identical complaints that regularly occur during spring due to environmental factors such as dust and pollen.

There was no evidence to suggest a difference in either the number of people attending an emergency department, or the reasons for attending an emergency department during or after the spray programme.

Concern that HIV positive people or people with other immunosuppressive diseases may be vulnerable to infection with BTK was considered and examined. The examination of all isolates of Bacillus species from all the hospitals and laboratories demonstrated that during a spray period, many people were exposed to BTK. The bacterium was easily recovered from a broad range of body sites. Despite this, when critical specimens including blood, body fluids, and tissues were examined by taking into consideration both the culture result and the patient information, we were unable to find a single case where BTK was a pathogen causing infection. Since all significant cultures collected during the period were examined, we conclude that no cases of infection in immunosuppressed persons have occurred during the time of the spray.

The study provided us with information associated with large occupational exposure to BTK. Although some of the ground spray workers remained culture positive for prolonged periods of time, most individuals remained culture positive for little more than a few days. Almost two thirds of the exposed workers developed some symptoms as compared to a one-third of control group of individuals who were not exposed. Many of the complaints included transient irritative effects such as eye, nose, and throat irritation, dry skin, and chapped lips. Complaints were more likely to develop in individuals who had a prior history of allergies. While a few cases of diarrhea or other gastrointestinal were reported, they were fewer in number than those reported within the control group. Importantly, there were no days of work loss attributable to exposure to BTK.

The information from the ground spray workers may seem to be contradictory to the physician sentinel and the emergency department studies , in that we were more able to detect health effects in exposed ground sprayers. It is important to appreciate that the amount of exposure that these workers had was considerably more than anyone in the general public might experience. Based on microbial air sampling studies that were done, many of the workers had exposures that were 500 times that which the general public

would have experienced, even if they had been directly exposed during an aerial fly-over. Exposure would have been substantially less for individuals who were indoors during and after the spray.

We demonstrated on two occasions that the public is readily exposed to BTK through consumption of commercially available fresh vegetables either organically or conventionally grown.

Although the number of complaints of gastrointestinal disease was small, in none of the cases, either from the ground spray workers, or from the general public where we recovered BTK in the stool did we find any evidence of a history suggestive of food poisoning, or a disease with watery diarrhea similar or suggestive of Bacillus cereus.

The study acknowledges that it did not study all individuals at all times. We have little information on those individuals with pre-existing problems with allergy or asthma who were made unwell by exposure to the spray but chose to not call the government MOTH-LINE, or attend a physician or hospital. Nonetheless it can be said with confidence that no cases of infection were detected in any hospitalized patients, or in any patients studied with bacterial cultures. Finally we found no evidence by examining individuals attending emergency departments that there was any measurable increase in serious community unwellness that could be attributed to the spray.

Background and Introduction

In spring 1992 it was decided by Agriculture Canada and the British Columbia Ministry of Forests that a combined aerial and ground spray programme was necessary to eradicate a recently found cluster of Asian Gypsy Moths in the Lower Mainland of British Columbia. The agent to be used for the programme was a commercial spray product known as Foray 48B produced by Novo Nordisk Bioindustrials, Inc. that contained the bacterium Bacillus thuringiensis var kurstaki (BTK). Although BTK sprays had been used for biological control by both the food and forest industries since 1971, and there was much evidence of its safety with respect to human health, there was sparingly little independent published information on its affects on human health when used to control insects in large urban area. One previous study, reported in 1990, had been laboratory based only, and as such, was unable to address individuals who did not attend a physician, or those who did attend a physician or emergency department but presented with complaints such as allergies, or asthma for which a microbiological culture was not considered as necessary for diagnosis. Also it was not designed to examine for health effects associated with different doses of exposure.

To address the wider aspects of the health effects of BTK spray on a large urban population the following study was developed. The study was done by university based researchers, funded by a grant-contract from the Ministry of Forests. The investigators performed their study independent of Agriculture Canada, Ministry of Forests, and of Novo Nordisk Bioindustrials. By studying many different population groups and environmental factors simultaneously it was possible to develop a wide, and thus reliable picture of the effects of BTK spray.

The body of this report is divided into 6 component chapters that reflect the different populations and targets for surveillance. Each chapter describes a series of experiments that represent one of the areas of focus. Each chapter is structured with an explanatory introduction, followed by a representation of the results of the experiments, which are in turn followed by discussion and interpretation of the results. The final section of each chapter will describe the methods and design of the experiments.

Food and Supplies Microbiology.

Addresses culture of the spray material and food sampling studies during and after the spray period.

Asian Gypsy Moth Telephone Hot-line Surveillance Programme

Monitors the telephone calls made to the government MOTH-LINE as a measure of the health concerns of the general public before, during, and after the spray.

Ground-spray Workers Surveillance Programme

Monitors the health effects of Foray 48B formulation of BTK spray in individuals who had occupational exposure many times more than the dose experienced by general public.

Sentinel Physicians' Offices Surveillance Programme

Monitors individuals who attended a group of sentinel physicians' offices at any time during the period of aerial spray programme, and identifies all health complaints including both infectious and non-infectious concerns before, during and after the spray. This phase of the study examined the frequency at which individuals could be clearly identified as being exposed to aerial spray, and observed for associations between symptoms and exposure.

Medical Laboratory Surveillance Programme

Examines all clinical isolates of BTK recovered from laboratories in the lower mainland during and after the spray period. Through this programme, all individuals, either seen in physicians' offices or in hospitals and suspected of being infected with BTK would be identified.

Lower Mainland Emergency Department Surveillance

Programme monitors admissions to emergency departments to examine for any increased incidence of severe asthma, allergy, or other complaints may be associated with aerial spraying.

Ground-spray Workers Surveillance Programme

Introduction

Between 18 April and 30 June 1992 the Asian Gypsy Moth Eradication Project Office supplemented the aerial spraying of the biological insecticide Bacillus thuringiensis variety kurstaki (BTK) around the Port of Vancouver, with ground application of BTK to areas at high risk of infestation from the Asian Gypsy Moth (AGM). Approximately 850 hectares of public parkland, residential, and industrial properties were ground-sprayed by licensed pesticide contractors and audited by Agriculture Canada personnel. The commercial BTK product Foray 48B, previously approved by Health and Welfare Canada for aerial and ground-spray application in urban areas, was used.

The objective of this study was primarily to determine if there were adverse health effects associated with the occupational use of the commercial BTK preparation Foray 48B, and secondarily to determine if persistent carriage of BTK occurred in association with adverse health effects.

Results

Populations

The study population, 120 ground-spray workers (GSW) (Table 1), had a higher proportion of males than did the control group (76% vs 59%), had more males in the 20-29 age group, and fewer females in the 30-39 age group. None of these differences were statistically significant. Smoking status was significantly different in the two populations - one third of the subjects were smokers while only 3% of controls smoked. However when smokers and non-smokers were compared regarding frequency of symptoms, no significant difference was evident between the groups. The populations were also similar with regards to pre-existing health status.

Microbial air sampling

Levels of BTK collected by personal monitoring exhibited ranges in keeping with varying conditions and spray patterns. The two cassette readings for an individual were averaged to represent the level in the breathing zone area localized between the cassettes. Results derived from the personal exposure monitoring are displayed in Table 2.

Exposure levels of samples taken simultaneously within a team generally followed a predictable relationship consistent with observed differences in spray patterns or conditions. For example, in a contractor A team the spray operator level usually exceeded four fold that of the auditor, but in a contractor B team only 1.5 fold difference was observed between sprayer and auditor levels. Spraying down from high lifts, spraying low foliage or spraying with prevailing breezes, resulted in lower exposures to spray operators than did

spraying upward into trees. For other team members, proceeding in parallel to sprayers yielded consistently lower readings than did being underneath overhead spraying or following behind the spray crew.

Mean exposure values of the groups ranged from 3,000 to 5.9 million organism spores per cubic meter of sampled air; four of the groups had comparable exposures. It is of interest that public relations personnel who followed crews through the sprayed West-End residential and downtown industrial areas had considerably higher exposure (7 fold) than those preceding the teams, and nearly 2 times higher than their auditors. However because these public relations personnel frequently interchanged positions, their mean exposures become equivalent with those of their auditors.

For those individuals who worked most shifts during the complete spray period, estimated cumulative BTK exposures range from a high of 720 million organisms (sprayers), to a low exposure of 5.4 million organisms (Kromacote card handlers).

Health Effects

Frequencies of adverse symptoms reported in questionnaire #2 by controls and GSW are shown in Table 3; 63% of GSW but only 38% of controls reported adverse health effects (any cause) occurring during the project period . GSW reported symptoms attributable to Foray 48B exposure at frequencies 2-3 times control levels of symptoms in all systems except gastrointestinal and headache which occured less frequently than in controls. Differences are statistically significant. The health effects following Foray 48B exposure appear to be generally transient and irritant in nature; dry skin, chapped lips, eye redness itch and burning, `runny nose' and nasal stuffiness were reported in approximately equal numbers. A number of individuals noted that their symptoms occured only briefly at the beginning of spray periods and when the Foray 48B spray concentrations were increased.

No significant or serious health problems resulted from Foray 48B exposure. Controls reported time lost from work due to illness during the spray period at a mean frequency of 0.16 days per individual and GSW reported an average 0.14 days per person.

Symptoms attributed by the workers to Foray 48B exposure were compared and no significant differences were found with respect to gender or smoking status. History of asthma, seasonal allergies, or eczema appeared to influence the frequency of reported post-exposure symptoms. Only 37% of the individuals with any of these pre-existing health problems (atopic individuals) reported no

effects from exposure to the ground spray, compared to 53% of individuals without allergies, asthma, or eczema. This difference is also statistically significant.

It was found that degree of exposure to Foray 48B spray also influences symptom frequency. As seen in Figure 1 there is a direct relationship between exposure level and reported symptoms. When compared to the low exposure category, a 1.7 fold increase of symptoms is reported in the medium exposure group, and 2.7 fold increase is seen in the high exposure group.

Carriage of BTK

All workers upon joining the cohort provided nose swabs prior to their exposure to ground-spray. Eight Agriculture Canada employees were culture positive on their initial swabbing. All were involved either with dispensing bulk BTK concentrate or recall sniffing from a container of Foray 48B which was opened during an orientation session so that employees could familiarize themselves with the odour of the product. All eight reverted to culture negative status during the project or within 30 days of project completion.

Although not all employees were available during all subsequent swabbing, many were swabbed at all four intervals. Of the 112 workers employed for the duration of the project and who provided at least one nose or throat swab, only 8 remained culture negative; 2 were supervisors intermittently in area of ground-spray activity, and the other 6 were on advance notice teams which were not directly exposed at any time to ground-spray. It was observed that BTK could be recovered from individuals (N=10) who had visited the spray zone for as little as ten minutes, if nose swabs were taken within a few days of exposure but not if taken after a one-week interval since last exposure.
Kromacote card team members were likewise culture positive the day of aerial spray but reverted to culture negative status during the 10-12 day interval between aerial sprays. Nearly all tested workers exposed to higher concentrations for several shifts (5-20) retain BTK for at least 5-6 days and most were culture positive for 14-30 days.

Follow-up GSW results (as of yet incomplete) indicate that few, and usually only those with large and repeated (>4 wk) exposures, retain BTK for longer than four weeks following their last BTK exposure. To date, of the 120 cohort study population, 24 subjects have been unavailable for follow-up culture, and of the remainder, who were swabbed following an interval of 30 to 63 days since last BTK spray exposure, 13 workers yielded BTK from nose swabs but none from throat swabs. All 13 positive on follow-up are male, four had low, and 9 had medium-high exposure levels; 72% of them reported sinus or throat irritation

attributed to Foray 48B exposure. Otherwise no consistent association could be found with regards to age, smoking status, or pre-existing health status.

Discussion

Study & Control Populations

Controls were comparable to subjects in age, gender, prior health history, and working outdoors in areas where commercial pesticides (not BTK) were utilized, but not in smoking status. However smoking was seen not to influence frequency of symptoms within the spray group. The main difference that could possibly influence outcome was that in contrast to the controls, many GSW worked at night and sometimes for double shifts. Length of work day, working outdoors and in shifts was unusual for many and contributed to some of the reported symptoms. Although the workers were asked to differentiate between the effects from Foray 48B spray and from other factors, reporting was subjective and may have been biased toward overestimating effects due to the spray.

Exposure Assessment

Personal monitoring equipment functioned reliably during the study. Dual individual readings proved useful for examining intra-individual differences and in more closely determining breathing zone levels than would have been possible using only one cassette per individual. Relative exposures estimated by observation corresponded closely with those determined by quantitative bioaerosol sampling.

Although the range of exposure levels within groups described in Table 2 is wide, levels of samples collected simultaneously on individuals and within teams were consistent with estimates made during observations of the spray operation, and were generally predictable with regards to relative levels for each team position.

Differences between groups could also be traced to differences in terrain and spray patterns. BTK dilutions were identical, area coverage and high pressure spray equipment was comparable, but contractor A sprayers spent less time spraying downward than did contractor B sprayers. Being exposed to more overhead spraying and having to follow behind the spray operation rather than parallel to it, were the most likely reasons that auditors and public relations personnel working with contractor B crews had higher relative exposures than did their counterparts with contractor A crews.

BTK Carriage

Little information is available in the published literature regarding human BTK colonization following exposure. Nasal swabs were found to be a relatively

sensitive method of detecting recent exposure to ground and aerial spray, but BTK was infrequently recovered from throat swabs.

Methodology necessary to demonstrate colonization as distinct from prolonged carriage was not available during this study. It was therefore not possible to determine whether BTK was recovered from nasal hairs or from nasal mucosa, or if the organisms were recovered in the spore form or in the vegetative state. In this study it was found that in the absence of subsequent BTK exposure, the organism was generally not recovered after an interval of one week following single exposure, but repeated occupational exposure to BTK over several weeks duration appeared to prolong carriage of BTK. Although it is theoretically possible that the BTK organism can persist in spore form for prolonged periods, it can be surmised that positive culture status persisting for more than 30 days after last BTK exposure represents colonization of nasal or sinus mucosa, but this is not proven. Individuals exhibiting persistent BTK carriage are being followed to determine when reversion to culture negative status occurs.

No discriminating features could be found to differentiate those who reverted to culture negative status within 30 days following last exposure to the BTK spray, from those who retain BTK for longer periods. Although the majority of the latter group attributed symptoms of `runny nose' or nasal stuffiness to their exposure to Foray 48B spray, no consistent differences could be found compared to other individuals who reported the same symptoms but who retained BTK for fewer than 30 days. Further information may be obtained from the 24 workers from whom follow-up results are still outstanding.

Health Effects

Quantitative exposures, measured in organisms per cubic meter of sampled air, represent exposure to BTK and also represent exposure to the other components found in Foray 48B. There appears to be a direct relationship between degree of ground spray exposure and frequency of symptoms attributed to that exposure. Most of the complaints, including eye nose and throat irritation, dry skin, and chapped lips, generally appear to be transient and irritant in nature. The Foray 48B label cautions that inhalation or contact with eyes and skin should be avoided but gives no specific information regarding adverse effects which can be expected from exposure to the product. Although certain bacterial cell wall components are known to be irritating and sometimes sensitizing to skin and mucous membranes, no data in the published literature could be found which differentiates what proportion of the observed irritation arises from exposure to the BTK organism, from that, if any, attributable to other components in Foray 48B.

It is not known what reduction of these adverse health effects could have been achieved by the wearing of more protective equipment. Face masks and goggles were not commonly worn for several reasons. Goggles tended to fog quickly which caused significant vision impairment at night and was felt by some to be responsible for a few near accidents. Workers also felt comfortable with the relative safety of the product. The individuals who wore face masks during most of shifts reported no fewer symptoms than the average for their exposure group, but the number of subjects (n=4) is too low for statistical comparison. Personal monitors worn by members of the Kromacote card team during aerial spraying demonstrated that exposures resulting from the occupational use of Foray 48B during the ground-spray program were up to approximately 500 times higher than those to which a member of the general public would have been exposed if outside and directly under aerial spray. Despite the exposure of the ground spray workers to relatively large quantities of BTK organisms (up to 720 million organism spores), no significant health problems were identified, and there were no days of work loss attributable to BTK exposure. This result is consistent with the good safety record of BTK product use over many years in North America.

Methods

Study & Control Populations

Employees from Agriculture Canada, BC Ministry of Forests, Mt. View Tree Service Ltd (contractor A), and Bug Busters Pest Management Inc. (contractor B), involved in the AGM Eradication Project formed a cohort of ground-spray workers (GSW) which was followed prospectively to determine if exposure to BTK resulted in carriage of the organism (in the nose) and was associated with adverse health outcomes.

Volunteer horticulture workers from BC Ministry of Agriculture Fisheries and Food, and students and faculty from Simon Fraser University, Biological Sciences (Pest Management), were included as a control population.

All members of the cohort received a briefing from the principal investigator regarding the study and known medical aspects regarding the BTK organism, signed a voluntary consent form, and completed an initial health survey questionnaire.

This surveillance project was observational only; exposure and conduct of employment (wearing or not wearing protective equipment etc) was determined by employer and employee, in accordance with provincial regulations.

Exposure Estimate

Due to differences in terrain covered by the two contractors, patterns of ground-spray application differed somewhat. Turbulence and wind tunnel effects resulting from uneven building height in the downtown industrial and residential West-End areas necessitated contractor B using mechanical lifts to treat all foliage to the full tree height. Spraying in these areas proceeded in tandem along adjacent blocks, and teams of six (two spray operators, two hose handlers, one record keeper-driver, and a foreman) advanced two high-pressure spray units along each block simultaneously spraying both sides. Two auditors proceeded with the sprayers, and the public relations personnel would both precede and follow behind the crews to control traffic and alert pedestrians. Sprayers and hose handlers would generally trade roles during a shift or on alternate shifts; public relations personnel would likewise exchange front and back positions.

The other contractor was required to spray only to the height reached by high-pressure hoses, and did not exchange positions. Teams consisted of one spray operator, one hose handler and one auditor. The one public relations individual preceded the crew often by several blocks, and was only infrequently required to follow behind the spray operation.

Foray 48B was distributed in bulk, diluted with water, and delivered as a high-pressure spray aerosol. During the first ground spray cycle, a dilution of 200 parts water with 1 part Foray 48B (200:1) was used. During subsequent cycles it was determined that better coverage was achieved when a much finer spray of higher BTK concentration was utilized. During the project, dilutions were reduced to 100:1, then 85:1, and finally 75:1. During the last spray cycle a trailer mounted jet turbine aerosol generator (Rotomister) replaced the high-lift units in the parks and the downtown area.

Relative exposures of workers to Foray 48B were estimated after direct observations were made of each job, and of various contractor teams, types of equipment and spray patterns. Auditor records were kept for each spray unit itemizing exact duration of spraying, area coverage, names, and role of each team member. These records were utilized to ascertain precise duration of exposure to ground spray for each participant in the study.

Quantitative Exposure Assessment

Microbiological air sampling (bioaerosol sampling) was conducted simultaneously within teams. Nucleopore polycarbonate filters sealed in plastic filter cassettes were connected to dual high volume constant flow portable air pumps, and were attached in the individual breathing zone of the worker. Pump

volume and exact exposure time for each individual was recorded. Mean exposure values were calculated and compared for significant differences; jobs having exposures that were not significantly different were grouped, and an average exposure was calculated for that group of jobs.
A team of individuals involved with monitoring aerial spray coverage was also studied. Completeness and density of aerial spray coverage was monitored in part through the use of Kromacote cards which were laid out in predetermined areas and retrieved following aerial spray. The six individuals responsible for the cards were generally exposed to air-delivered aerosol during the fly-over and for the two or more hours it took to collect the cards. Individual breathing zone samples were also collected.

Cumulative individual exposure to Foray 48B was calculated by multiplying the mean exposure value assigned to the individual's job (described above) by the actual hours each worker was exposed to spray operations. Workers were then grouped into three exposure categories (low, medium, and high). Exposure in controls was categorized as nil.

Carriage of BTK was determined by the growth of BTK colonies from nose and throat swabs. Swabs were taken before the ground-spray, at the end of the first spray cycle (12-48 hrs post-exposure), before the third spray cycle (5-6 day interval since last exposure), and following last exposure to BTK (3-5 wks, depending on availability of subject to follow-up); individuals showing persistent carriage received an additional swab approximately one month later.

Adverse Health Effects
Two questionnaires were administered in order to identify adverse health effects. Associations between frequency and type of reported adverse health effects, and prolonged carriage of BTK were examined, and differences were tested for statistical significance.

Table 1

1992 Vancouver AGM Ground-Spray
Workers Surveillance Project
Population Variables

	GS Workers N=120	**Controls** N=29
Mean Age (yrs)	31	34
Smokers (%)	33	3
Non-Smokers (%)	67	97
Male (%)	76	59
Female (%)	24	41
* Atopic (%)	36	39

* Indicates past history of any of Asthma, Seasonal Allergies, Eczema

Table 2

1992 Vancouver AGM Ground-Spray
Worker Surveillance Project
Bioaerosol Exposure *

Position	Samples (No.)	Range	Mean	Standard Deviation
contractor A				
sprayer	8	0.9 - 10.5	5.9	3.3
hose	6	0.6 - 4.0	1.7	2.0
auditor	4	0.5 - 3.2	1.4	1.2
public relations	2	0.6 - 0.7	0.7	0.05
contractor B				
sprayer	30	0.6 - 15.8	3.1	3.1
hose	16	0.2 - 8.3	2.2	2.2
auditor	30	0.4 - 9.6	2.0	2.3
public relations	10	0.2 - 6.9	2.0	2.2
Kromacote card	3	0.2 - 0.6	0.3	0.3

* Bioaerosol Exposure expressed as millions of BTK organism spores per cubic meter of sampled air

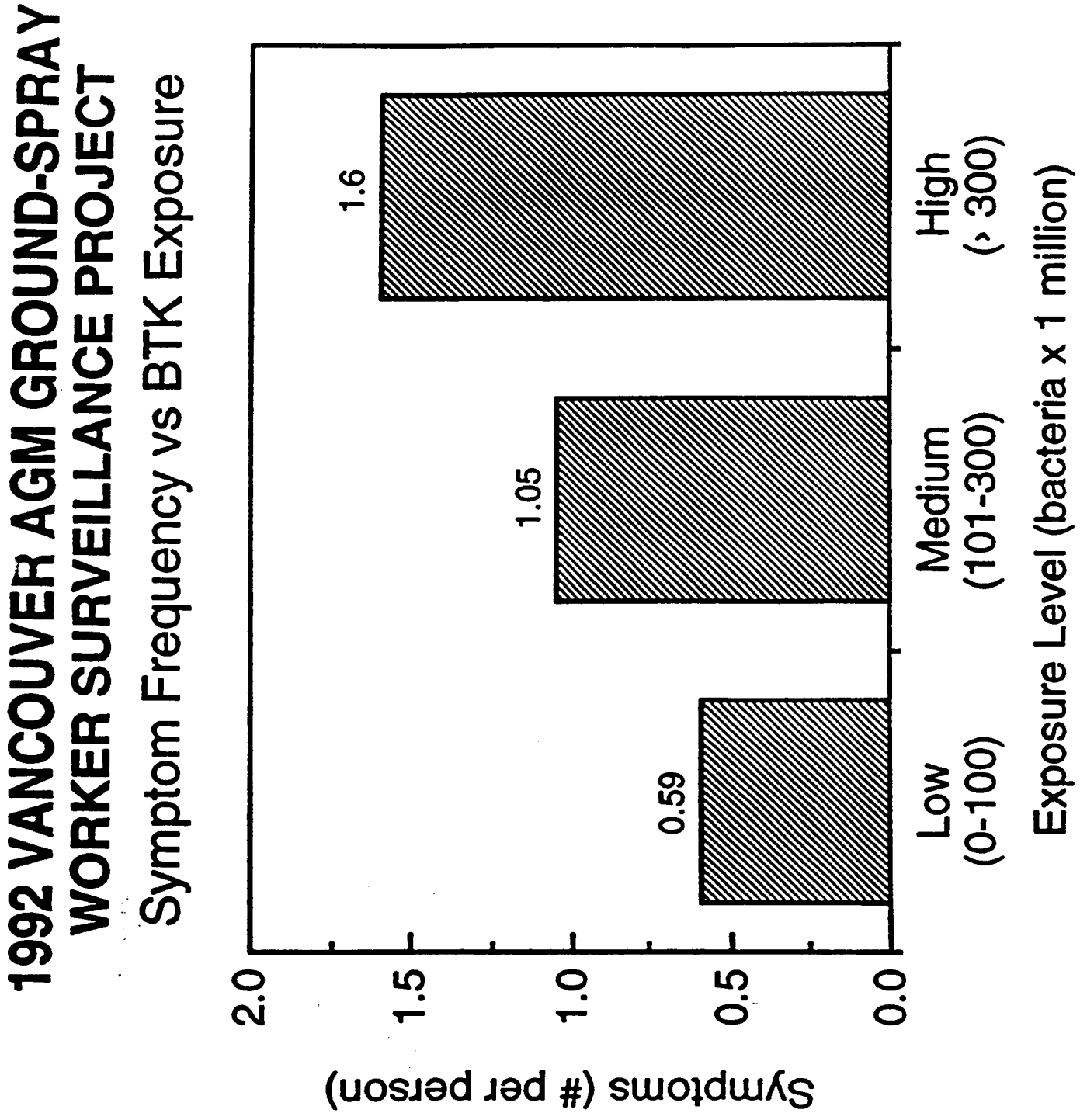
Table 3

1992 Vancouver AGM Ground-Spray Workers Surveillance Project
Post Exposure Effects
(Reported Symptoms, Percent)

Complaint (site)	Controls [a]	Ground-Spray Workers [b]	
	Total %	Total %	BTK Attributed *
Any Complaint	**38**	**63**	**55**
Skin (Dry Itchy) / Chapped Lips	10	34	25
Eyes (Redness Itch Burning Puffiness)	13	20	19
Headache	10	7	3
Throat (Dry, Soreness)	7	29	22
Nose (`Runny') / Sinus (Stuffiness)	13	27	17
Respiratory (Cough, Tightness)	3	20	12
Digestive (Nausea, Diarrhea)	10	7	4

[a] Controls N = 29
[b] Workers N = 120
* (%) = Effects attributed (by the workers) to BTK exposure

Figure 1


1992 VANCOUVER AGM GROUND-SPRAY
WORKER SURVEILLANCE PROJECT
Symptom Frequency vs BTK Exposure
Symptoms (# per person)
2.0
1.5
1.0
0.5
0.0
0.59
1.05
1.6
Low
(0-100)
Medium
(101-300)
High
(> 300)
Exposure Level (bacteria x 1 million)

Asian Gypsy Moth
Telephone Hot-line
Surveillance Programme

Introduction

To provide a centralized information service to the public regarding the 1992 Vancouver Asian Gypsy Moth Eradication Project, Agriculture Canada established a toll-free telephone hotline in January 1992. A survey was conducted of the health-related calls received between 15 April and 15 June 1992, an interval encompassing the four aerial spray periods.

Results

A total of 26,406 hotline calls were received between 27 January and 30 June 1992 (figure 1). Frequency of calls reached a peak of 15,523 in April 1992, including a high of 1,795 in one day. Only a fraction (8.6%) were health-related, and these reached a monthly maximum of 1,736 calls in April (figure 2). Requests for health risk information accounted for many calls during the weeks preceding the first aerial spray, but as seen in figure 3 the proportion of health-related calls, which reached a maximum of 24% during the week preceding the first aerial spray, dropped sharply after aerial spraying commenced on 18 April, and reached a low of 1% during the fourth spray period.

Of the 19,893 calls received during the period 15 April - 15 June, 939 (4.7%) were health-related. The latter were surveyed, and of 321 calls where information was recorded, 74 were from individuals requesting information only, were from callers outside the spray zone, were unrelated to the aerial spray or were simply protesting the project. The remaining 247 calls represented complaints from individuals who reported being within the spray zone or exposed to spray, and who attribute their symptoms to that exposure; the systems affected are listed in table 1. Seasonal allergy-like symptoms and those of the "flu-like" complex accounted for 36% of these complaints. A further 17% involved respiratory complaints and one-half of these described asthma symptoms.

Discussion

The AGM hotline was particularly busy during April and May 1992, when more than 24,000 calls were logged. However only 8.6% of the total hotline calls, and only 4.7% of calls received in the surveyed interval, were health-related. In the weeks leading up to the first aerial spray, several features critical of the proposed AGM eradication project appeared prominently in the media and may have contributed to the apprehension in many of the individuals who called the hotline during March and April seeking health risk information. The AGM hotline was thus able to provide callers a medium through which concerns could be ventilated, and from which accurate detailed information was readily available. A relatively sharp decline in health-related calls was noted after the spray program commenced, which appeared to reflect the absence of serious medical complications arising from exposure to the spray. Furthermore not all

those who called during the surveyed interval which encompassed all four aerial spray periods were concerned about the effects of the spray. 23% requested only information, and many of those reporting symptoms stated they were doing so only because they understood all symptoms occurring during this project regardless of cause were to be reported to the mothline.

Due to lack of planning time and limited resources only a relative few of the health-related calls were recorded in detail, and no randomly-controlled comparative study was possible. Furthermore no regional baseline data regarding frequency of common symptoms exists, and no information is available regarding either occurrence of symptoms or lack of effects in the vast majority of individuals within the spray zone who did not phone the hotline. Likewise there is no data with which to adequately compare callers, to those individuals living outside the spray zone and not exposed to aerial or ground spray. Although some interesting observations were made in this passive survey, few conclusions can be made from this information regarding the effect of exposure to aerial and ground spray during the AGM Eradication Project.

Methods

During the survey interval telephone calls were fielded by regular hotline personnel, who because of time constraints, found it possible to record details of health-related calls only in the relatively few non-busy periods. None of the operators were health care providers, and none had received special instruction in health-related interviewing, Thus information was gathered in a non-systematic, non-random fashion, and varies in content and in degree of completeness.

In addition to regular personnel, three physicians staffed phones during the three days prior to the first aerial spray and thereafter on spray days to provide specific medical advice and assessment to referred calls.

It should be noted that in this passive survey, callers were self-motivated to report their information and were not matched to controls. Furthermore no comparative study of health effects occuring in individuals living within and outside the spray areas was conducted.

Table 1
1992 Vancouver AGM Hotline: Surveyed
Health-Related * Calls 15 Apr-15 June

Complaint (N = 247)	**Percent**
Seasonal Allergy Symptoms	19
"Flu-like" complex	17
Headache	6
Eyes	2
Nose / Throat	10
Respiratory	17
Gastrointestinal	7
Skin	8
Other (Multiple Systems)	7

* Primary reason for call

Figure 1

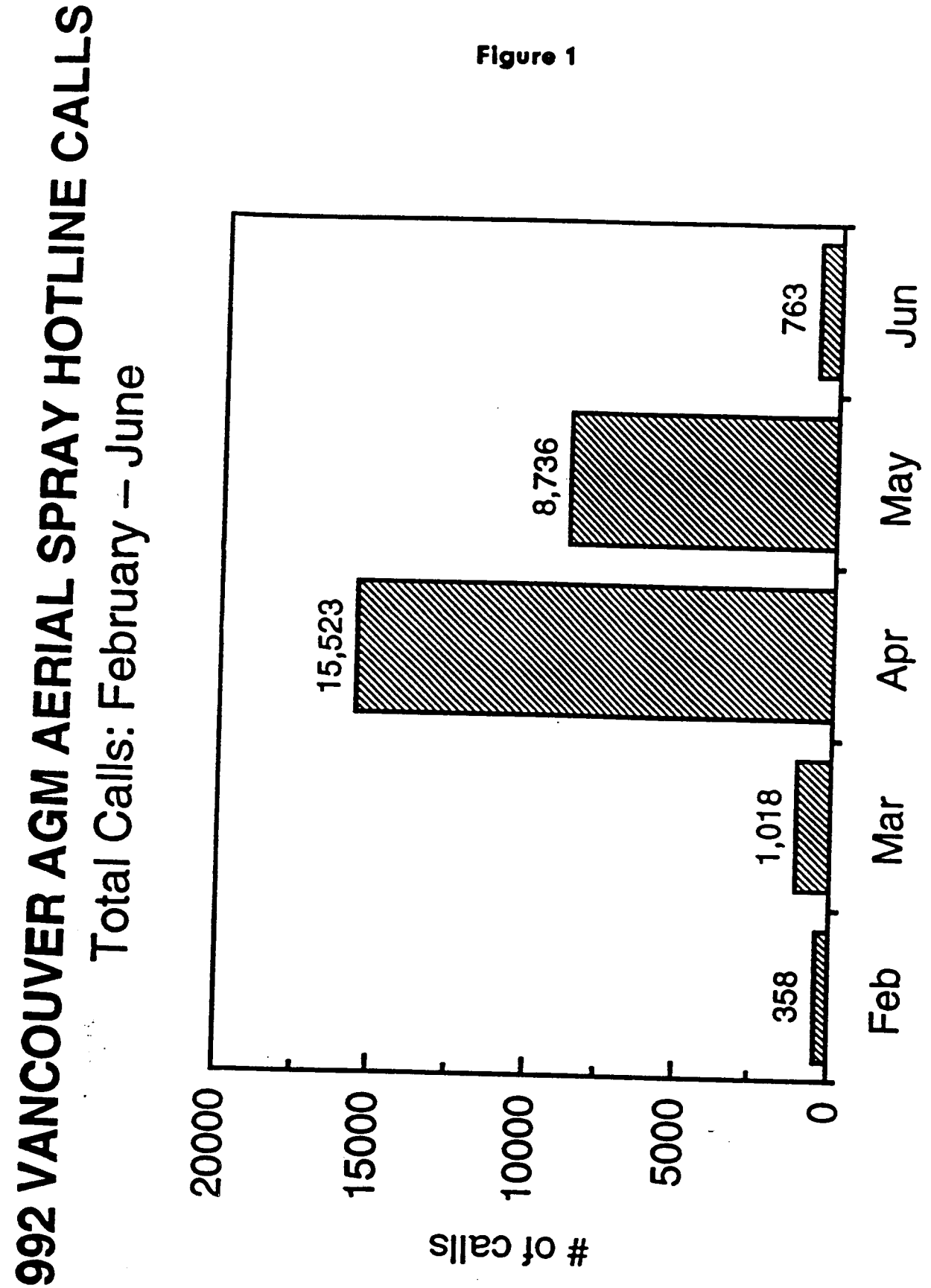

1992 VANCOUVER AGM AERIAL SPRAY HOTLINE CALLS
Total Calls: February – June
# of calls
20000
15000
10000
5000
0
358
1,018
15,523
8,736
763
Feb
Mar
Apr
May
Jun

Figure 2

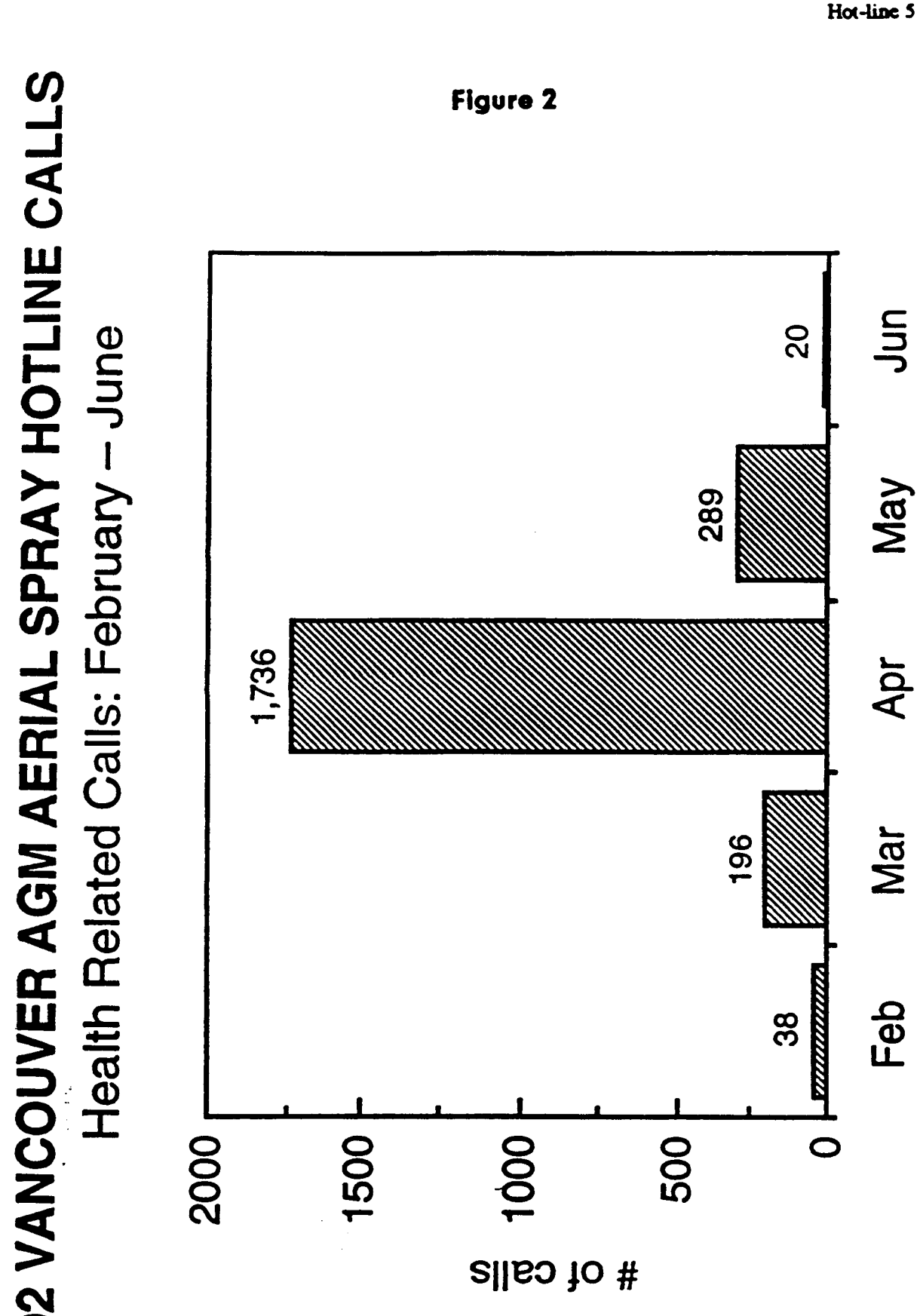
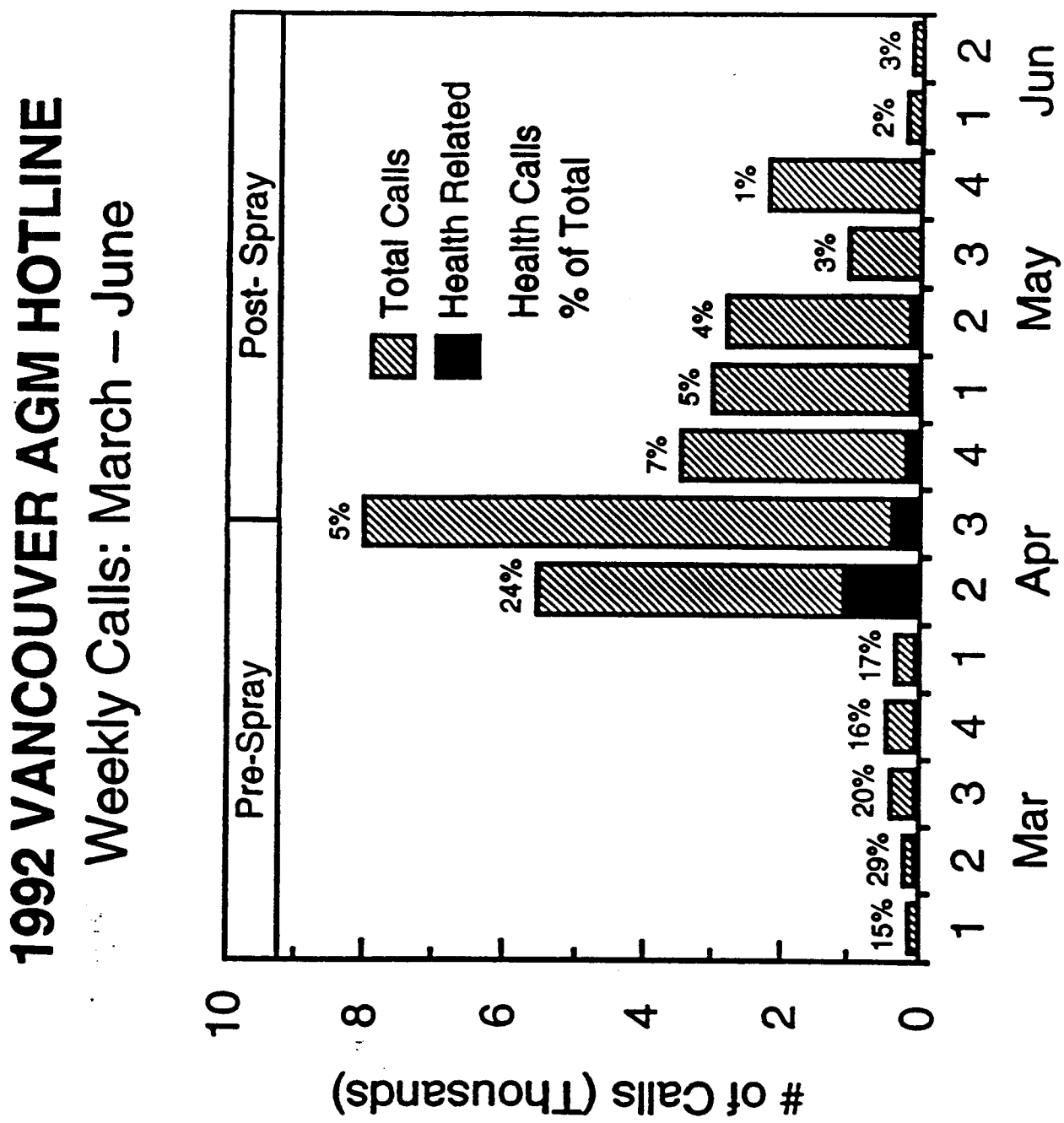

1992 VANCOUVER AGM AERIAL SPRAY HOTLINE CALLS
Health Related Calls: February – June
# of calls
2000
1500
1000
500
0
38
196
1,736
289
20
Feb
Mar
Apr
May
Jun

Figure 3


1992 VANCOUVER AGM HOTLINE
Weekly Calls: March – June
Pre-Spray
Post- Spray
Total Calls
Health Related
Health Calls
% of Total
# of Calls (Thousands)
0
2
4
6
8
10
15%
29%
20%
16%
17%
24%
5%
7%
5%
4%
3%
1%
2%
3%
1 2 3 4 1 2 3 4 1 2 3 4 1 2
Mar
Apr
May
Jun
Weeks

Sentinel Physicians' Offices Surveillance Programme

Introduction

The physicians' office surveillance programme examined a broad component of the Lower Mainland community to identify those individuals who had been exposed to the aerial spray containing Bacillus thuringiensis var kurstaki (BTK). Demonstration of having been exposed to the spray was sought by testing for the presence of BTK in the individual's nose or throat. Links between being culture positive and having symptoms were then examined. A group of 29 family practice physicians associated with the Department of Family Practice, Faculty of Medicine, University of British Columbia who have their office practices spread throughout a wide geographic area in the Lower Mainland was asked to participate in the study.

Results

Twenty-four of the original 29 physicians were able to participate in the study. One thousand one hundred forty patients were admitted into the study. The number of specimens submitted ranged from 10 to 95 per physician.

Characteristics of the total patients demonstrated a gender ratio females:males of 1.9:1 with mean age for females 38.2 years and for males 36.4 years. The most prominent age group was between 20 and 40 years for both genders. These characteristics are typical of patient populations attending family practice offices.

Postal code information was used to determine if the person lived in or outside the areas covered by the aerial spray programme. Postal codes were available for 1085 (96%) patients , and covered a broad range of postal regions including North Vancouver, West Vancouver, Vancouver, and Burnaby. When postal zone maps were compared against the flight pattern maps, it was determined that 566 (49.6%) of individuals had their residence within the spray zone while 519 (45.5%) lived outside. For the remaining 55 (4.8%) no postal zone was provided.

One hundred twenty eight (11.2%) of the nose specimens cultured from the patients were found to contain resembling BTK. Seventy four (57.8%) were from individuals living within the spray zone, while 50 (39.1%) were from outside the zone. The remaining 4 (3.1%) were from those for whom no postal code information was available. The difference in culture positivity rate for those living within the spray zone (13.1%) as opposed to those outside the spray zone (9.6%) was not significant (O.R.=1.41, 95% CI=0.96-2.06).

A clear timing pattern was noted linking culture positivity to aerial spraying (see figure 1). In each instance, culture positive specimens were tightly clustered around the time of spraying. In each instance, with the exception of the first fly-over, the rate of positivity dropped off on sequential days until it ceased to be detected. With the 4 individual spray periods considered, the longest period of positivity detection following spraying was 5 days.

Neither gender, nor any age group, nor any geographic region was more likely to be culture positive (Table 1).

Reasons for attending the physician are recorded in figure 2. Six hundred seventy five were either asymptomatic or had symptoms unrelated to the spray. The remaining 465 (40.8%) had symptoms including allergies, asthma, rhinitis, conjunctivitis, otitis media, sinusitis, headache, pharyngitis, upper or lower respiratory tract infection, shortness of breath or skin rash. No individual symptom group was more likely to be culture positive when compared against the rate in asymptomatic individuals. Because of the design of this phase of the study, it is not possible to determine with certainty if any of these symptoms were more common in those living inside or outside the spray zone, however, it was noted that for those for whom postal code information was available, and a possible complaint was noted, 240 (54.1%) were from those living within the spray zone, and 204 (45.9%) were from outside. This proportion was markedly similar to the proportion of individuals living inside and outside the spray zone strongly suggesting that possible complaints were no more likely in those living inside the spray zone, as compared to those living outside.

A follow-up letter was written to each physician to make inquiries about each of the patients with a positive culture. It was noted that 19 of 128 patients with BTK (14.8%) were treated with antibiotics as were 167 of 1012 patients without BTK (16.5%). None of the patients with BTK had a negative outcome even though 119 (85.3%) had received no antibiotics, and of the 19 who did, 6 who had been started on antibiotics to which the organism was highly resistant. Of the 19 patients with BTK who had been started on antibiotics, 9 lived within the spray zone, 10 did not. Four had been started on antibiotics prior to the onset of the spray programme. No link between diagnosis, possible exposure to BTK, and decision to prescribe antibiotic therapy could be identified.

Discussion

The results of the physicians' office surveillance provides information about the ease in which the nose was able to trap BTK suspended in air, and also the short duration of nasal carriage of the organism, and also the lack of any apparent relationship between the timing of the spray and the types of complaints presenting in the physicians offices. There is little indication that BTK was associated with significant infection considering that the vast majority either of people did not receive an antibiotic, or if they did, only one third received an antibiotic to which the organism would respond. If infection was present, it would have had to have been brief and self limited.

The difference in pattern for positive cultures during the first fly-over may have been the result of the intermittent days of flying as opposed to the sequential flying days of the other periods.

The lack of association between the nose cultures being positive and the postal code information suggests that either the tests used for microbial identification were sufficiently non-specific to ensure that the organisms were all the same, or other factors such as location of work, occupation, or location at the time of spraying also play a role in whether or not an individual could become exposed to the aerial spray. Because serotyping of strains was not available, the former is a distinct possiblity; however,the clear timing of positives with the timing of the aerial spray-overs strongly argues for the latter.

Methods

The physicians were requested to enroll the first five patients to come to their office during the five days prior, during and after each spray period, regardless of their symptoms or complaints, and in addition to collect swabs from any patients who came up to 10 days after each period with any symptoms "of interest" that may be related to the spray including respiratory infection, pharyngitis, asthma, allergy, sinusitis, conjunctivitis or rash. The physicians would test for the presence of BTK by swabbing either nostril for bacterial culture. In addition, if a significant infection was thought to be present, a second culture would be sent to a medical laboratory as a method for clinical detection.

To ensure timely processing of cultures, and information, all materials were collected and transported by study personnel to the Microbiology Laboratory of University Hospital, Shaughnessy site. All swabs were accompanied by a form providing the person's age, postal code, symptoms, diagnosis, and whether they were to be put on antibiotics.

Swabs were tested by standard culture techniques using 5% sheep blood agar and nutrient agar. Cultures were identified by typical colonial morphology, and were confirmed as Bacillus thuringiensis by characteristic spore and crystal morphology. Bacillus thuringiensis strains were defined in conjunction with 2 known postive strains of BTK using an abbreviated biochemical scheme. Strains were defined as BTK if they were beta-haemolytic, lecithinase positive, urease positive, aesculin hydrolysis positive and failed to ferment mannose. Serotyping was not available at the time of the study.

Information and culture results were encoded in a computer database software programme and were analyzed by comparing the numbers of those with symptoms of asthma, allergy, respiratory symptoms, conjunctivitis, or rash against those who were either asymptomatic or were presenting with unrelated complaints. Variables examined included, patient characteristics (age, gender), home postal code as a measure of whether their place of residence had been exposed to spray, date of onset of complaints and date of presentation at their physicians' office. Tests of statistical analysis including odds ratio, or chi-square analysis were applied to group differences in order to determine if differences were sufficiently large to not have occurred by chance alone.

Table 1

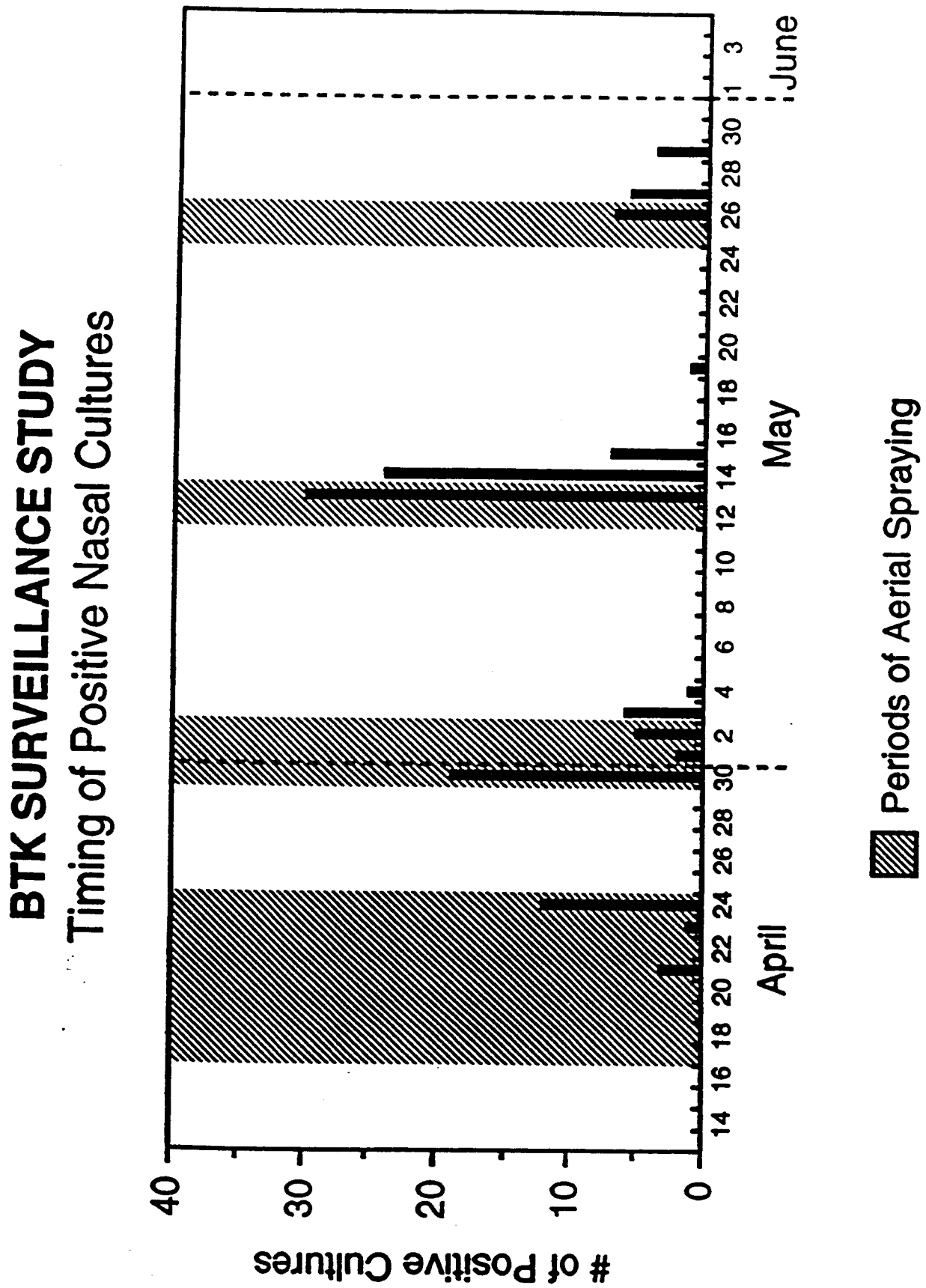
Physicians' Office Surveillance Programme
characteristics associated with culture result

		Culture Negative	Culture Positive
Gender *			
Male		333 (86.5%)	52 (13.5%)
Female		659 (89.9%)	74 (10.1%)
Residence Location *			
Inside spray zone		492 (86.9%)	74 (13.1%)
Outside spray zone		469 (90.4%)	50 (9.6%)
Age *			
range (years)	0- 20	148 (85.5%)	25 (14.5%)
	21- 60	668 (88.9%)	83 (11.1%)
	61-100	159 (90.9%)	16 (9.1%)
mean (years)		37.9	36.1
"Of interest" symptoms reported **			
Yes		425 (91.4%)	40 (8.6%)
No		587 (87.0%)	88 (13.0%)

* **Block does not include all 1140 cases, because some data not provided.**

** **See Text for definition of "of interest" symptoms**

Figure 1


BTK SURVEILLANCE STUDY
Timing of Positive Nasal Cultures
# of Positive Cultures
0
10
20
30
40
14 16 18 20 22 24 26 28 30 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 1 3
April
May
June
Periods of Aerial Spraying

PHYSICIAN SURVEILLANCE PROGRAMME

Complaints Reported: April 13 - June 4, 1992

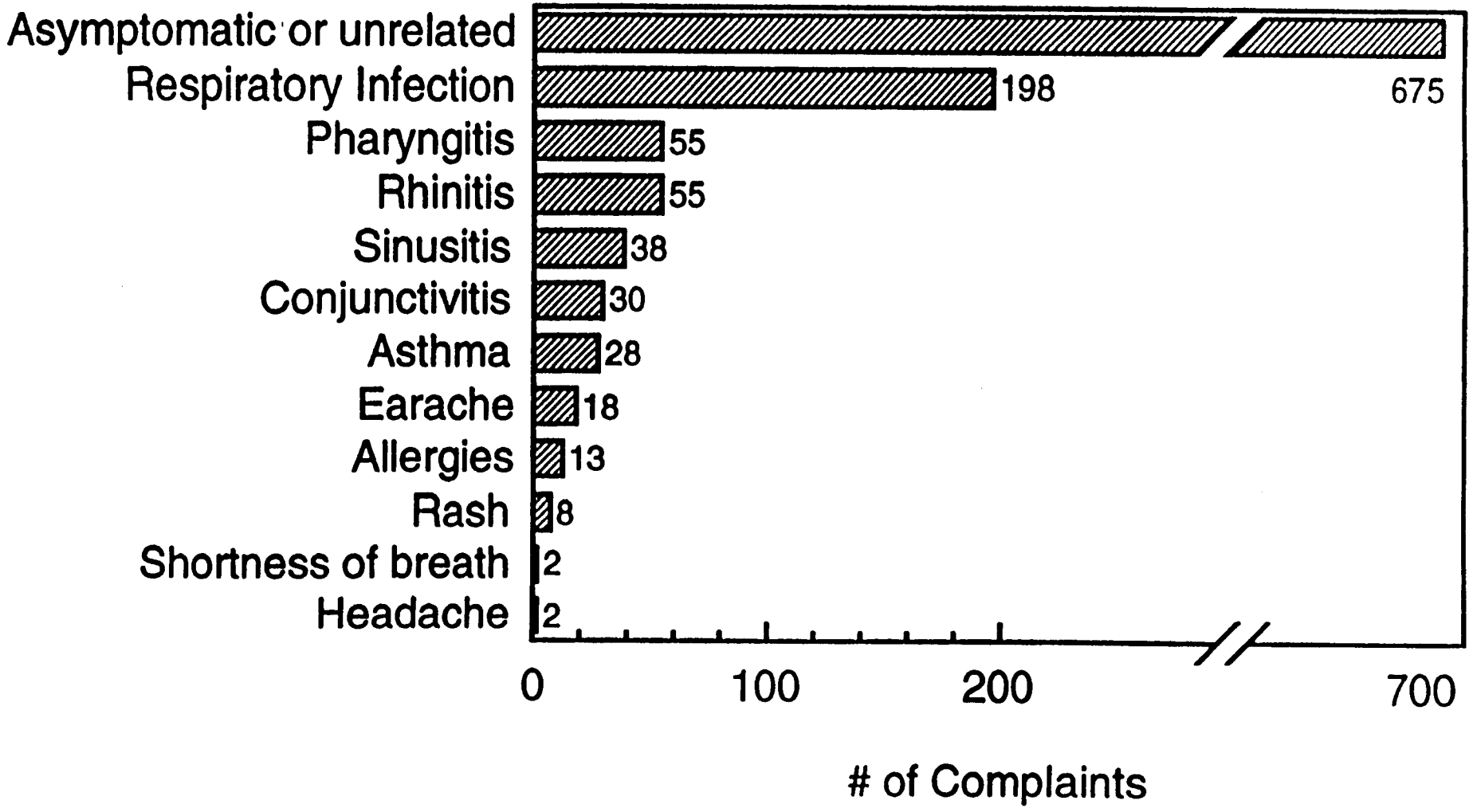


Figure 2

Sentinel Physicians 8

Medical Laboratory Surveillance Programme

Introduction

The objective of the laboratory surveillance programme of the study was to capture and identify all isolates of BTK recovered from any patient in the Lower Mainland who presented to a physician either in an office practice or in a hospital with sufficient symptoms that a specimen was collected which would identify a possible infection. This would provide an index of those patients who had developed a clinical infection associated with BTK.

Results

Through the cooperation of Lower Mainland laboratories, 429 bacterial isolates were submitted for analysis. Three hundred twenty-five (75.8%) isolates were characterized as BTK. In 43 of these 325 cultures (13.2%) BTK was recovered in pure culture. I n 42, the growth was characterized as light. In 1, it was characterized as heavy. Further clinical information was sought on the patients for these 43 cultures. Along with these cases, information was sought for 2 invasively collected specimens, one body fluid, and one tissue biopsy, both which had mixed culture results. Information was available for 37 of the 45 cases (82.2%).

Seven isolates were recovered from blood cultures or intravascular lines. Following close examination of each case, in none of the cases was BTK the causative agent of patient's unwellness. In two of the 7 cases, the patient had a rapid recovery while treated with an antibiotic to which the bacterium was highly resistant. In two the patient had a transient mild fever episode, which resolved although neither was treated with antibiotics. In 1 case, the culture was collected as a test of cure following completion of antibiotic therapy given in hospital for a previously well documented bacterial infection involving a different organism. When the culture was collected, the patient was feeling well, without fever or any other signs of infection. He was discharged from the hospital the next day. In the remaining 2 cases, swabs for microbiology cultures were taken of 2 intravascular lines at the time of their removal. In both instances, culture of the swab was found to contain BTK even though the patient was afebrile and feeling well, and discharged from hospital. In neither case was BTK or another Bacillus species recovered from cultures prior to removal of the

lines. It was determined that in all of these instances, the BTK was coincidental and probably represented skin contaminants either from the patients themselves or from hospital ward or laboratory staff.

Two specimens were from tissue biopsies. In one, the BTK was found to be coincidental to a definite diagnosis of viral infection. In the other, the organism was from a patient in whom infection was not the suspected diagnosis, and an alternate definitive tissue diagnosis was made. In each instance the organism was from broth culture only, and was suspected to represent a coincidental contaminant.

In 3 patients BTK was recovered from an aspirated body fluid, one from a knee, one from pleural fluid, and one from peritoneal fluid. In none of the cases was bacterial infection considered to be the clinical diagnosis. In each instance, the culture was found in broth culture only, and was interpreted as a coincidental skin contaminant.

Thirteen of the pure culture isolates came from genital-urinary cultures. Of 12 urine cultures, 6 were from symptomatic patients and 4 were from patients with screens for asymptomatic bacteriuria. Of the symptomatic patients, 3 improved without antibiotic therapy, 1 improved while receiving antibiotics to which the organism was highly resistant. One of the remaining two had onset of symptoms 4 days before the onset of spraying. The other lived, far outside the spray zone. Of the asymptomatic patients, one culture was positive while the patient was on doses of an antibiotic to which the organism was highly sensitive, and therefore, most likely represented a skin contaminant rather than true pathogen. The other was not treated, did not develop into symptomatic disease, and was not found to be positive on a subsequent culture. The single patient with a culture found to be in heavy pure growth was a woman with symptoms compatible with a urinary tract infection, however all symptoms resolved without antibiotic therapy. One individual was found to have light pure growth cultured at the time of a gynecological procedure. Infection was not a considered diagnosis. Clinical information was unavailable for 2 patients with light pure growth in urine cultures.

Ten of the pure culture isolates came from cultures of eyes. Two patients had symptoms resolve while on common eyedroppers that contain antibiotics to which the organism is sensitive. Neither patient had related their unwellness to spray exposure. A third patient did not respond to antibiotic therapy to which the organism was highly sensitive. A non-infectious diagnosis was made by a consultant ophthalmologist. The patient responded to appropriate therapy. The remaining patients had symptoms resolve without antibiotics. Clinical information was unavailable for 2 patients with light pure growth in an eye culture.

Nine of the pure positive isolates came from skin cultures. Clinical information was available for 5, all of which were of doubtful clinical significance. The sites included neck, hand, foot, chest. In each a non-infectious alternate diagnosis was considered, and in each situation, the problem resolved without antibiotics.

In one case, a couple was exposed to ground spray while cycling. Both experienced facial moisture but no discomfort. A nose culture collected the day following exposure from one, and was found to grow a pure and heavy growth of BTK. No culture was collected from the other. No infection of inflammation developed in either person. Neither was treated with antibiotics.

Along with information taken from patients with pure culture results, additional information was also sought by standardized questionnaire from the attending physicians on 16 patients with positive faecal cultures. The questionnaire asked specifically about symptoms and associations with foods. Information was available for 12. Eight of the patients had transient diarrhea, while 4 were collected from patients without diarrhea for other reasons. One patient was noted to have a known bacterial enteric pathogen and 3 had known enteric parasites. No pathogens were identified in any of the others. With the exception of one patient, who associated symptoms with curried chicken, no associations with any particular foods were noted. No patient related their symptoms to contact with either aerial or ground spray. No information was collected on a control group of patients.

Discussion

Because all laboratories in the Lower Mainland participated in this phase of the study, any true infection that occurred during or after the spray period and was made by documented culture would have been captured by the study. For that reason it was determined that the population at risk would be the total population of the Lower Mainland. Prior census data has reported the population to be approximately 1,400,000.

While information was not available on all cases where BTK was recovered, it is important, that all cases where blood, body fluids, biopsies, and intravascular lines were investigated. No cases that fit the study criteria for infection were found. In this regard, the results of this phase of the study are markedly similar to those reported from Oregon, where the vast majority of positive cultures were thought to represent culture contaminants, or at most, minor local site infections.

Concern that HIV positive people or people with other immunosuppressive diseases may be vulnerable to infection with BTK was examined indirectly. All isolates of Bacillus species recovered from all hospitals and laboratories during the period of the spray programme were examined by the study laboratory. Despite this, when all critical specimens were examined from both clinical and laboratory perspectives, we were unable to find a single case where BTK was a cause of infection. Since all significant cultures collected during the period were examined, we conclude that no cases of infection in immunosuppressed persons have occurred during the time of the spray.

The large number of positive enteric, genital and urinary cultures was noted. The possible source for these positive cultures all represent coincidental contamination from the collection or processing of the cultures. It is also possible that they represent true enteric isolates that have successfully passed through the gastrointestinal tract, and have become part of the genital flora. To definitively answer these questions, one would require further study.

It is important to appreciate that the recovery and identification of most of these positive cultures occurred only as a result of the laboratories' cooperation with this study. In most instances, the presence of occasional Bacillus species colonies in urine, faecal, or skin cultures would be overlooked as an inconsequential component of mixed normal flora. For this reason, it is not possible for the study to say if the enteric, faecal, or skin isolates detected at this time represent any change from normal findings.

It is appreciated that the absence of infection does not exclude cases of irritation that may have occurred because of contact with spray. Indices of irritative symptoms were monitored through the BTK ground-spray workers, Physician's Office and Lower Mainland Emergency Department Surveillance Programmes.

Methods

All Microbiology Laboratories receiving specimens in the Lower Mainland were asked to assist with the study by providing all isolates of Bacillus species recovered from clinical specimens for species identification, and to provide information including the site from which the specimen was recovered, the amount of Bacillus in the specimen, whether the Bacillus isolate was recovered in pure culture, and if not whether it was associated with normal flora for the site, or associated with a known pathogen. All isolates were referred to University Hospital UBC site where each isolate was identified. The methods for identification were the same as those described in the physicians' office surveillance programme.

For those isolates identified as BTK recovered either in pure culture or in heavy amounts, additional clinical information was sought in order to determine if the culture was likely to represent an infection with BTK. In accordance with ethics approval by the University of British Columbia, a letter was written to the ordering physician requesting information on the clinical case. The letter was followed by a telephone conference with a registered nurse associated with the study.

The definition of infection used for the purposes of this study included the following criteria:

1: the patient presented with signs compatible with an infection

2: BTK was recovered either in pure culture, or in heavy concentration in the absence of a known pathogen

3: If the patient was treat with an antibiotic, the outcome of the infection was consistent with the expected result based on sensitivity testing. The infection would not be expected to respond to an antibiotic against which BTK was highly resistant, but would be expected to respond to one to which BTK was sensitive.

Table 1

Sites from which BTK was recovered

Site	Pure Culture	Mixed Culture	Total
Genital	1	55	56
Urine	12	22	34
Eye	10	17	27
Faeces	0	16	16
Leg-foot-toes	1	10	11
Head-neck	1	8	9
Chest-abdomen-back	3	6	9
Arm-hand-fingers	3	6	9
Skin (not specified)	1	5	6
Nose-throat	1	7	8
Sputum	0	6	6
Blood	5	0	5
Body Fluids	2	1	3
Biopsy tissues	1	1	2
Intravenous lines	2	0	2
Total	43	160	203

Lower Mainland
Hospital Emergency Department
Surveillance Programme

Introduction

Visits to the Emergency Departments of six hospitals in the Greater Vancouver region for the period April 13, 1992 to June 4, 1992 were reviewed as an indirect measure of serious unwellness that may have developed in the community as a consequence of the aerial spray of Foray 48B.

Results

Six Emergency Departments and hospitals gave permission for the research assistants to come into their departments, review records and abstract the information. Five of the departments were either within the spray zone or bordered the spray zone. The sixth department was more removed from the spray zone but nonetheless did have patients whose home address was within the spray zone.

Over this 53 day interval, spraying occurred on 12 days. Four of the 12 spray days and 11 of the 41 non-spray days (including Victoria Day, Monday May 18) were weekend days. Seven of the 11 non-spray weekend days occurred in the post spray interval.

The total number of visits to the six emergency departments was 33,636. The pattern of visits is diagrammed in Figure 1. The average number of visits on each day was 634.6 with the range extending from 457 to 807. The maximum number on a spray day was 732 and the minimum was 503.The average number of visits on Spray days was 613 while the average number on non-spray days was 640.9. This difference was not statistically different.

The influence of the weekend on the number of visits to the emergency departments was marked. To control for the effect of the weekend, the number of visits on a spray weekend was compared to the number of visits on non-spray weekends. The average number of visits on a spray weekend day was 652 while on a non-spray weekend day it was 724.

A 10 per cent sequential sample of the emergency department visits resulted in a review of 3,517 records. The residence of the patients, as determined by the postal code, was outside the spray zone for 2,473 and in the spray zone for 1,044. The overall ratio of non-spray zone patients to spray zone patients was 2.4:1. The pattern of a greater number of visits on weekends while

present, particularly for residents of the spray area, was not as pronounced. Table 1 summarizes the average number of visits which occurred. The average number of visits during non-spray days was not different from the average number of visits on spray days. Similarly no difference was found when the number of visits on a spray weekend was compared to the number of visits on a non-spray weekend.

Of the 3,517 patients in the 10% sample 1625 were females and 1891 were males. The sex of one patient was not recorded. The ratio of females to males was not different when patients from spray areas were compared to non-spray areas.

The age range of the 10% sequential sample extended from 2 weeks to 101 years. The mean age for patients coming from non-spray areas was 34 while from spray areas it was 33.6.

Over the 53 day span approximately 3,000 medical records were reviewed to determine if the patient experienced any of the "of interest" problems. Of this group, 1,839 had a discharge diagnosis referable to the eyes or respiratory tract or otherwise consistent with the list of diagnoses described in the methods section. The majority, 1,352, were for respiratory problems. One hundred eighty three (183) had eye complaints, 25 had an unexplained fever, 60 with an unexplained allergic reaction, another 100 had a rash and 119 had a nosebleed.

The respiratory conditions were reported as asthma in 536 patients, bronchospasm or wheeze in 33, pneumonia in 147, bronchitis in 205, an upper respiratory tract infection in 287, a viral illness in 13 and an exacerbation of chronic obstructive lung disease in 81. The remainder had a variety of respiratory diagnoses.

Of the patients with eye complaints 4 were identified as having corneal ulcers. Three of the four were females, two had a residence within the spray zone but neither presented at the time of spraying. One of the remaining two presented at the time of spraying. One of the four was a contact lens wearer. All were discharged home and cultures were not obtained on any.

As with the total visits the number of "of interest" visits to the emergency departments with problems was greatly influenced by the day of the week. Figure 2 graphically represents the number of visits for any of the "of interest" conditions . The average number of visits for any of the "of interest" conditions on spray days was 27.3 while on non-spray days it was 25. This difference was not statistically significant. When only the visits on weekends were considered the number was 35.5 on a spray weekend versus 32.8 on non-spray weekends.

Asthma was the presenting problem for 10 patients on each on the spray days and for 10.1 patients on the non-spray days. When the area of residence was considered, the number of asthma visits from people residing inside the spray area was 2.9 per spray day and 2.6 per non-spray day.

The frequency with which eye complaints presented to hospital was the same in the spray and non-spray periods. There were 3.4 visits per day during the spray periods and 3.5 per day on the non-spray days.

The number of presentations to the emergency department with a complaint of fever was on average less than one per day. In the spray period there were 0.8 visits per day while during the non-spray period the number of visits was 0.5 per day.

The number of patients admitted with allergies was 1.1 during both the spraying and non-spraying days.The mean number of visits for a complaint of a rash was 1.8 during the spray period and 1.9 during the non-spray period. Finally nosebleeds which presented to the emergency department was 2.5 per day during spray periods and 2.2 per day in the non-spray periods. In all these comparisons the differences were not statistically significant.

The ratio of non-spray area to spray area resident in the group identified as having the "of interest" conditions was 2.6:1. This ratio is similar to the ratio found in the sequential sample.

The ratio of female to male in the "of interest" group was 876:958 The sex ratio by area of residence did not change.

The mean age was 26.9 years with the range extending from two weeks to 101.7 years. The group of patients who had a postal code within the spray

zone had a mean age of 25.2 while the group whose postal code placed them outside the spray area was 27.5.

The total number of admissions to hospital was 211. One hundred ninety eight of the patients admitted to the emergency department with a respiratory problem were admitted to hospital. The range of visits extended from none through to 10 in one day. The mean number of respiratory visits on a spray day was 4.5 while on a non-spray day it was 3.5. When only visits on weekends were compared there were 5.2 visits/day on spray weekends compared to an average of 4.2 visits/day on non-spray weekends. These differences were not significantly different.

The number of admissions to hospital following presentation to the emergency department with the problem of asthma was 88. The average number on spray days was 1.8 and the average number on non-spray days was 1.6.

When comparisons were made between pre and post spray periods for the "of interest" conditions the average number of visits during the pre-phase was 32.9 per day and during the post-phase it was 35.2 per day. For patients from the spray area the number of daily visits in the pre-stage averaged 8.1 while in the post-phase it was 10.4. The differences were not statistically significant.

Discussion

A pattern of increased visits to emergency departments on weekends was evident. This pattern was present for all groups but it was attenuated when visits by spray area residents were examined. The pattern did not change when aerial spraying occurred.

When comparisons were made between the number of visits on spray days versus the number on non-spray days significant differences could not be found. Differences could not be found when pre spray admission rates were compared to post spray rates. Similarly when only visits for those whose area of residence was within the spray zone were considered, the pattern persisted and statistically significant differences were not present.

Explanations for not finding differences in the frequency of visits between the two periods are many. The first and the most obvious is that there was no change in pattern. Support for this conclusion is the apparent lack of concern in areas where aerial spraying has occurred in the past. Though formal studies have not been published on emergency room visits it is probable, that if there was a consistent effect, the effect would have been noticed, studied and published.

A second explanation is an effect was present but was so small that it could not be statistically measured in this time frame. In general the smaller the effect, the larger the number of observations required to demonstrate statistically that a change did occur.

The lack of a demonstratable effect on visits should not be interpreted as there not being any effects from the spray program. It is possible the people in the spray zone experienced more symptoms than did those in the non-spray zone but the symptoms were not severe enough to warrant going to an Emergency Department. It is possible that severe effects could have occurred, but at a very low frequency which would make it difficult to demonstrate.

Methods

Visits to the Emergency Departments of six hospitals in the Greater Vancouver region for the period April 13, 1992 to June 4, 1992 were reviewed. The information collected for each day included: the total number of visits, and the age,sex, and postal code for every 10th admission. The day books in each department were reviewed by one of two research assistants (both nurses) to identify patients with respiratory problems, conjunctivitis, corneal ulcers or problems which might be associated with a sensitivity reactions. During the initial period of reviewing the day books the research assistants worked together to develop a common list of problems and/or complaints which would result in further review. Once patients were identified as possibly having a condition of interest their emergency medical chart was obtained. Each chart was reviewed to determine the discharge diagnosis, the patients age, sex, postal code, and time of admission. Information was

also obtained on whether cultures were obtained and whether or not the patient was admitted to hospital or discharged home. This data was abstracted onto a predesigned form and was subsequently entered into a microcomputer.

The respiratory conditions included in the review included asthma, pneumonia, bronchitis, upper respiratory tract infections, and exacerbations of chronic obstructive pulmonary disease. Conjunctivitis and corneal ulcers were the two problems regarding the eyes that were reviewed. Patients with allergic reactions or a rash were included if no obvious reason for the reaction was noted in the chart. Patients with a fever, which could not be explained by any physical or laboratory signs at the time of admission to the emergency department were included. Finally patients with a nosebleed were included if there was no history of trauma.

The postal codes for the spray areas were determined by comparing the maps of the spray areas provided by Agriculture Canada to a Canada Post map of the Forward Sortation Areas (FSA, the first combination of 2 letters and one number) for the region. For the FSA straddling the spray zone the Local Delivery Unit (LDU) were obtained from the British Columbia Department of Finance. The postal code of the patients was compared to the postal codes of the spray zone to determine whether the patient resided in or out of the spray zone.

The dates of spraying were obtained from Agriculture Canada.

Comparisons of spray days versus non-spray days were of two types. In the first all days in which spraying did not occur were called non-spray days. In the second set of comparisons the non-spray days were categorized as either pre spray or post spray. The post spray period was defined as the 5 days after a spray period plus the days between spraying during the first spray interval. All other non-spray days were considered as pre spray.

Analysis used Odds ratios, T test, chi square and ANOVA as appropriate.

Table 1

The mean number of visits to Emergency Departments by spray area and non-spray area residents.

Group	Spray Area (mean)	Non-spray Area (mean)	Total (mean)
Sequential			
spray days	20.1	45.1	65.2
non-spray days	19.6	47.1	66.7
"Of interest" *			
spray days	10.0	26.5	36.5
non-spray days	9.4	24.7	34.2
Respiratory			
spray days	7.4	19.9	27.3
non-spray days	6.3	18.6	24.9

* See text for explanation of "of interest"

Figure 1

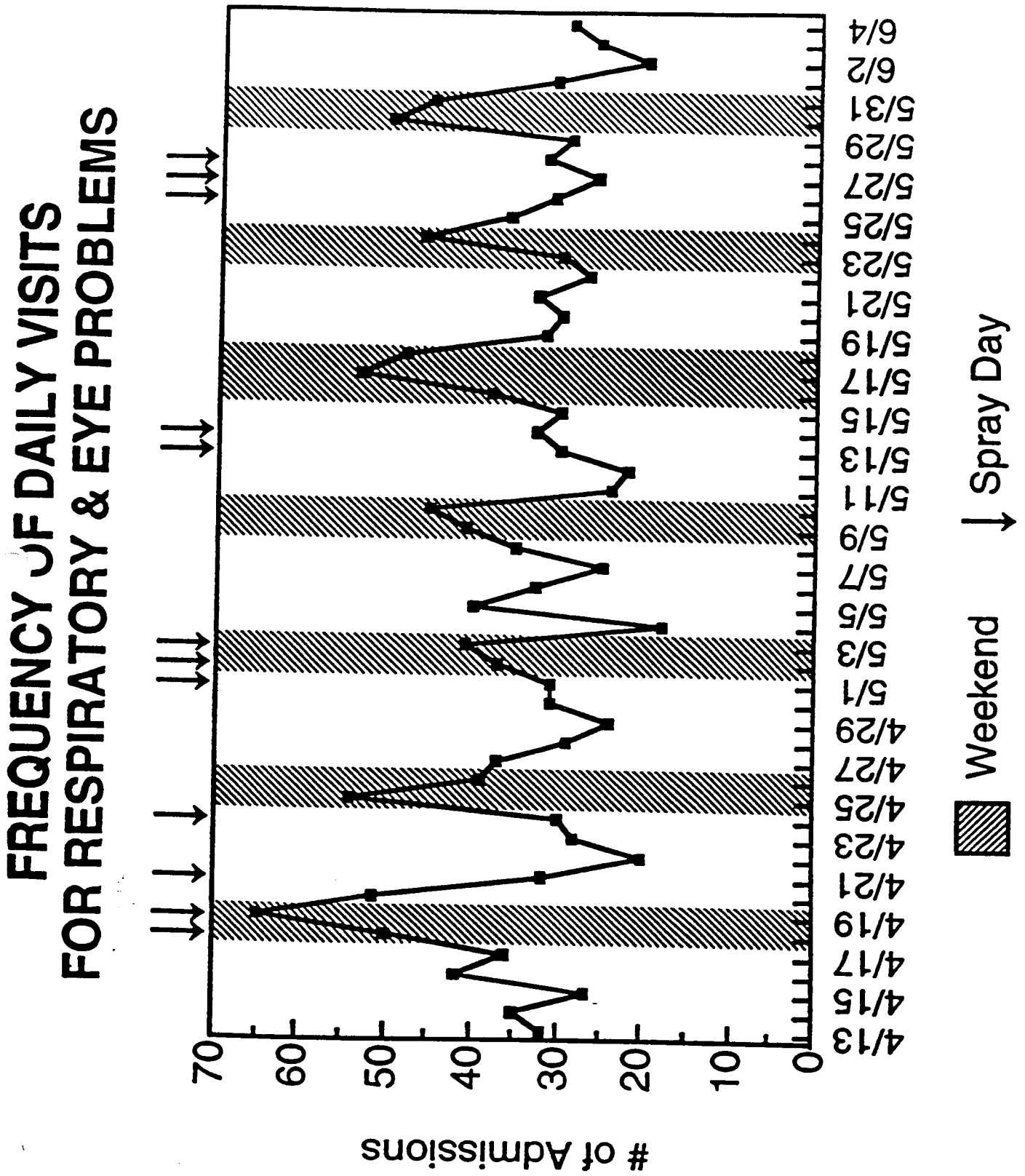
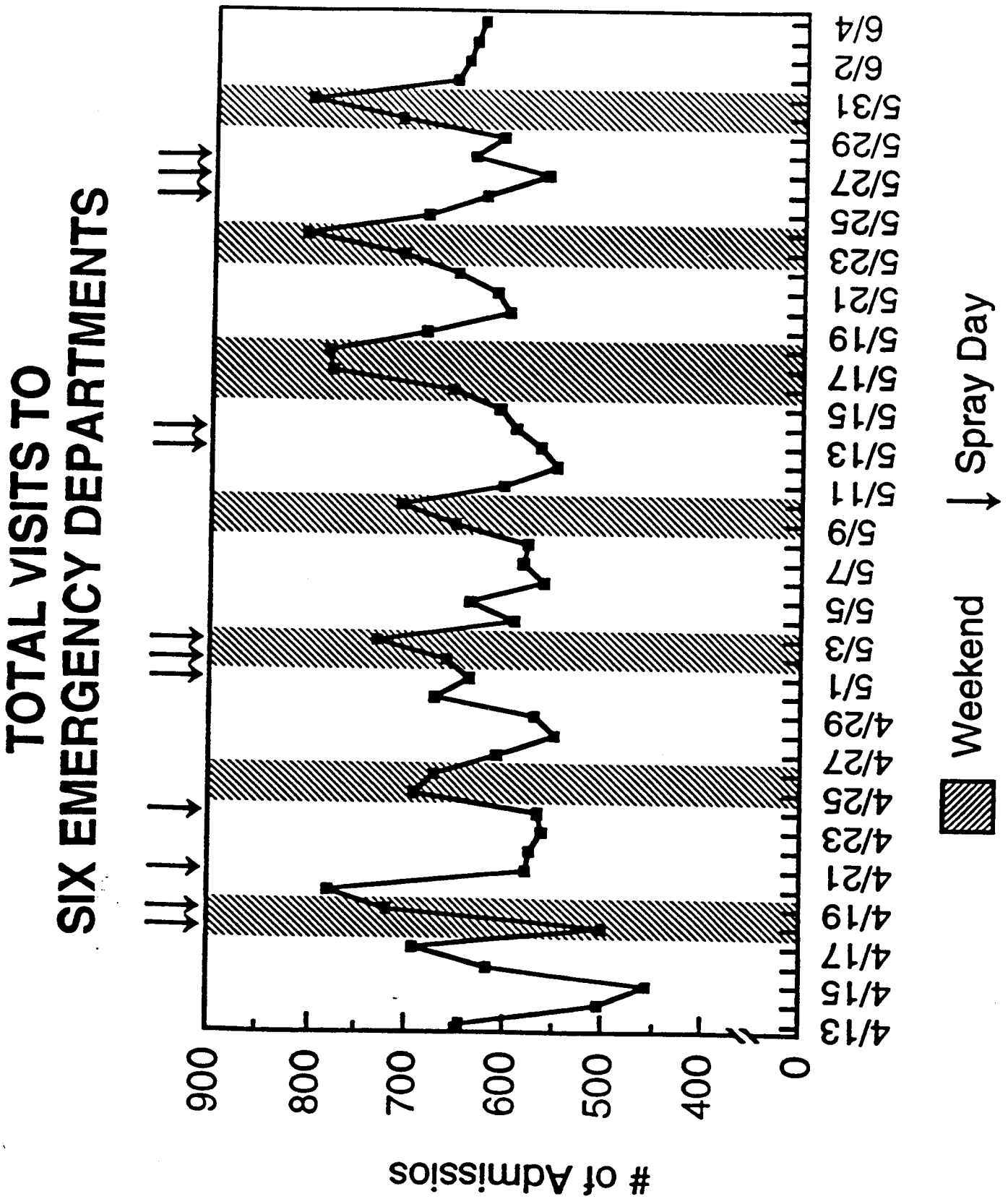

FREQUENCY OF DAILY VISITS
FOR RESPIRATORY & EYE PROBLEMS
# of Admissions
0
10
20
30
40
50
60
70
4/13
4/15
4/17
4/19
4/21
4/23
4/25
4/27
4/29
5/1
5/3
5/5
5/7
5/9
5/11
5/13
5/15
5/17
5/19
5/21
5/23
5/25
5/27
5/29
5/31
6/2
6/4
Weekend
Spray Day

Figure 2


TOTAL VISITS TO
SIX EMERGENCY DEPARTMENTS
# of Admissios
900
800
700
600
500
400
0
4/13
4/15
4/17
4/19
4/21
4/23
4/25
4/27
4/29
5/1
5/3
5/5
5/7
5/9
5/11
5/13
5/15
5/17
5/19
5/21
5/23
5/25
5/27
5/29
5/31
6/2
6/4
Weekend
Spray Day

Acknowledgments

The authors wish to warmly acknowledge the help and cooperation of the individuals , their offices and institutions who worked so diligently in the project to enable us to develop this report

- Ed van der Gugten
- Gunhild Jerabek
- Margaret Johnston
- Camille Leicester
- Scott Macrae
- Richard Mathias
- Chris van Netten
- Wendy Tan
- Terry Taylor
- Kay Teschke
- Carl Whiteside
- Family Practice Sentinel Physicians
- Metro-McNair Laboratories
- Biomedical Laboratories
- Burnaby Hospital
- Children's Hospital
- Eagle Ridge Hospital
- Lion's Gate Hospital
- Royal Columbian Hospital
- St. Paul's Hospital
- University Hospital UBC site
- University Hospital Shaughnessy Site
- Vancouver General Hospital
- Department of Biomedical Communications Art Division, University of British Columbia
- Department of Family Practice,
- University of British Columbia
- The Technical Reviewers

Food and Supplies Microbiology

Discussion

Culture of Foray 48B was performed because of previous concerns raised about possible contamination of the spray with other bacteria emanating from the environment. No such contamination was observed.

The results of the food phase of the study demonstrated that sources of bacteria resembling BTK but from sources other than the aerial and ground spray programmes were present during the study period. With the methods available it was not possible for us to distinguish the isolates from food from the isolates from the spray. It might be argued that the source of the isolates on the food was from the aerial or ground programmes, however their being found both during and after the spray period, and not on foods that were displayed side by side makes that most unlikely.

These food isolates provide a reasonable alternative to explain BTK in faecal and urinary samples submitted from a broad range of the participating laboratories.

Methods

The Foray 48B spray material was collected by individuals experienced in handling large volumes of material. It was examined microscopically and by culture on a range of selective and non-selective media, incubated both aerobically, and anaerobically for up to 72 hours. All plates were examined for the presence of organisms appearing different from the control culture of known BTK.

Samples of selected vegetables were examined for the presence of BTK. Food microbiology was performed in two fashions, the first by surface swab technique, and the second by a sample destructive technique. With the consent of individual store produce section managers, surface cultures of vegetables were collected by swab in 3 separate grocery stores. In each instance a sterile swab was vigorous rubbed against the surface of the vegetable sample. The swab was then returned to its plastic case and submitted to the microbiology laboratory for processing. In this first phase vegetables were collected from one store that deals exclusively with organically grown vegetables and one store that have a broader range of foods available.

Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy: I. Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens

Elide A. Pastorello, MD,[a] Cristoforo Incorvaia, MD,[a] Claudio Ortolani, MD,[b] Sergio Bonini, MD,[c] Giorgio W. Canonica, MD,[d] Sergio Romagnani, MD,[e] Alfredo Tursi, MD,[f] and Carlo Zanussi, MD[a]
Milan, Rome, Genoa, Florence, and Bari, Italy

Background: *The detection of specific IgE antibodies to environmental allergens does not always coincide with a diagnosis of clinically evident allergic disease, because some patients with positive skin and/or in vitro test results have no symptoms related to the allergen or allergens that induced the antibodies.*
Objective: *In a multicenter study the optimal cutoff values for specific IgE antibody levels and skin test results that could discriminate between patients with symptomatic and those with asymptomatic allergy were determined.*
Methods: *IgE antibodies specific for a panel of common aeroallergens were assayed with the Pharmacia CAP System (Pharmacia, Uppsala, Sweden) in two groups of patients, a group of 267 patients with symptomatic allergy and a group of 232 patients with asymptomatic allergy—both with positive skin prick test results—and in a group of 243 healthy, nonallergic control subjects. The cutoff values were established by receiver operating characteristic analysis.*
Results: *A significantly higher mean specific IgE antibody value was found in patients with symptomatic allergy compared with patients with asymptomatic allergy ($p < 0.001$) and in patients with symptomatic allergy compared with healthy control subjects ($p < 0.001$). The optimal CAP System cutoff value between patients with symptomatic and those with asymptomatic allergy was 11.7 kU/L, and when seasonal allergens were compared with perennial allergens, the cutoffs were 10.7 kU/L and 8.4 kU/L, respectively. The optimal cutoff value for the skin prick test was a wheal area of 32 mm^2 for seasonal allergens and 31 mm^2 for perennial allergens. The skin test had a lower diagnostic value (sum of sensitivity and specificity) than the CAP System.*
Conclusions: *Cutoff values for specific serum IgE antibody levels are likely to be useful in clinical practice to distinguish symptomatic from asymptomatic allergy in patients with positive skin test results. (J ALLERGY CLIN IMMUNOL 1995;96:580-7.)*

Key words: *Aeroallergens, specific IgE, symptomatic allergy, asymptomatic allergy, cutoff*

The pathophysiology of allergic diseases depends on a series of factors such as the releasability of mast cells and basophils and the responsiveness of target organs to mediators released from these cells. Still, the pivotal factor is undoubtedly the presence of specific IgE antibodies to environmental allergens.[1] The detection of these antibodies by in vivo or in vitro methods, nevertheless, merely

From [a]Allergy Center, First Department of Internal Medicine, University of Milan; [b]Bizzozero Division of General Medicine, Niguarda Ca' Granda Hospital, Milan; [c]Andrea Cesalpino Foundation, First Department of Internal Medicine, University "La Sapienza," Rome; [d]Allergy and Clinical Immunology Center, Scientific Department of Internal Medicine, University of Genoa; [e]Department of Allergy and Clinical Immunology, University of Florence; and [f]Department of Allergy and Clinical Immunology, University of Bari.

Received for publication May 2, 1994; revised Jan. 30, 1995; accepted for publication Jan. 30, 1995.
Reprint requests: E. A. Pastorello, MD, First Department of Internal Medicine, Pad. Granelli, via Francesco Sforza 35 20122 Milano, Italy.

0091-6749/95 $5.00 + 0 **1/1/63847**

Abbreviations used
ROC: Receiver operating characteristic
SPT: Skin prick test

indicates the existence of sensitization to given allergens; their presence does not always coincide with clinically significant allergic disease. Thus the diagnosis of allergic disease is usually based on a combination of information obtained from the medical history, which by itself cannot be considered as the "gold standard," with that obtained from in vivo and/or in vitro tests. The magnitude of the specific IgE response to a given allergen should theoretically be consistent with the clinical relevance of that allergen, but the correlation between the level of specific IgE antibodies and the clinical expression of an allergy has so far been unsatisfactory. In a series of studies that addressed this issue,[2] only a correlation between specific IgE level measured by RAST and severity of seasonal symptoms in patients allergic to ragweed has been reported.[3] This is probably because until recently, sufficiently accurate quantitative assays for IgE antibodies were not available. The need for quantitative assays to "facilitate better definition of the relationship between quantity of IgE antibody and the symptoms or risk of disease" was underlined in a recent Position Statement by the American Academy of Allergy and Immunology.[4]

In the practice of allergy, the physician must often identify the allergen or allergens to which a patient is or is not clinically sensitive. For this, we usually depend on skin tests or serologic assays. Problems occur when the patient's history is not definitive, in which case in vivo and/or in vitro tests may be especially useful, and when the results of the tests do not correlate with the clinical picture. We therefore designed a study to determine whether, in patients with evidence of allergic sensitization, the quantitative results of the tests would allow us to distinguish those patients with clinically significant allergic disease from those who have no clinical symptoms.

To that end, three groups of subjects were recruited. Patients in the first group had a positive skin test response to one of five common aeroallergens and a clear history of rhinoconjunctivitis or asthma, those in the second group likewise had a positive skin test response but clearly had no symptoms related to the corresponding allergen, and those in the third group were healthy nonallergic individuals. IgE antibody levels to the allergens being evaluated were quantitated with the Pharmacia CAP System assay (Pharmacia, Uppsala, Sweden), which has recently been reported to have excellent analytic sensitivity and specificity.[5-7] For a group of three common seasonal allergens and for house dust mite allergen, optimal IgE antibody cutoff levels were obtained from receiver operating characteristic (ROC) curves. From these values, sensitivity, specificity, and diagnostic values for the antibody assays were then determined and compared with values obtained similarly for skin prick test (SPT) results.

METHODS

Patients

Subjects were recruited in 22 allergy consultation centers in Italy (listed in the Appendix). Each center was asked to select between 20 and 40 subjects who had positive skin test responses to one of five common aeroallergens *(Phleum pratense, Betula verrucosa, Parietaria judaica, Dermatophagoides pteronyssinus,* and *Felis domesticus.)* To determine the subject's clinical status, a detailed allergy history was obtained from each subject by a trained allergist. In addition, patients had to respond to a standardized questionnaire with items covering the kind of symptoms, their occurrence, and the subject's age at onset of these symptoms. Only symptoms that were definitely present or definitely absent were counted; suspected or possible symptoms were not considered as evidence.

Group 1 consisted of 267 patients with symptomatic allergy (170 female and 97 male subjects; mean age, 27.1 years; range 8 to 59 years). These subjects had to have a definite history of or current rhinoconjunctivitis, asthma, or both, with a clear association between the occurrence of allergic symptoms on exposure to the same allergen that induced a positive SPT reaction. Patients were excluded from the study if they had a history of asthma of more than 2 years' duration (to avoid cases in which established inflammation might influence the patient's clinical status), if they had been treated with any form of corticosteroid or other immunosoppressive therapy during the preceding 36 months, if they had an elevated IgE antibody level caused by another disease, or if they had ever received allergen immunotherapy.

Group 2 consisted of 232 patients with asymptomatic allergy (125 female and 107 male subjects; mean age, 31.1 years; range, 9 to 54 years). These subjects had to have a negative history of rhinoconjunctivitis and asthma caused by the aeroallergens that elicited the positive SPT reaction. In all cases, they had been referred to one of the participating allergy centers for evaluation of symptoms caused by noninhalant allergens (e.g., milk)

or by aeroallergens clearly unrelated to those being evaluated (e.g., pollens of trees such as olive or cypress, which have short and precisely limited pollination periods). Special care was taken to exclude symptoms caused by any of the five allergens included in this study. To rule out allergy to house dust mites, respiratory symptoms could not be elicited on exposure to house dust; to rule out allergy to cats, patients must not have had an allergic reaction, including urticaria, on contact with cats. In cases of allergy to mites or cats, patients must also not have ever had perennial symptoms; any respiratory symptom that the patient might report had to be of strictly seasonal nature. To be considered asymptomatically allergic to the pollens, patients must not have had any respiratory symptom during the corresponding pollination period, that is, from February to April for birch trees, from April to June for the grasses, and from May to September for *Parietaria* species. Patients were also excluded from the study if they had been treated with any form of corticosteroid or other immunosuppressive therapy during the preceding 36 months or if they had ever received specific immunotherapy for the allergen eliciting a positive SPT reaction.

Control subjects

Group 3 consisted of 243 nonallergic subjects (150 female and 93 male subjects; mean age, 31.6 years; range, 9 to 59 years. These subjects were recruited in the same allergy consultation centers from the medical staff members and their siblings. They had to have a definitely negative history of hay fever, asthma, urticaria, atopic dermatitis, and any other significant immunologic disease. In addition to the above exclusion criteria for groups 1 and 2, all candidates for this group were screened for IgE antibodies with the Pharmacia Phadiatop test. This in vitro test detects specific IgE to a mixture of common aeroallergens and has been reported to be a useful method for confirming the absence of allergy in subjects with a negative allergy history.[8] Subjects with a positive result were excluded from the study.

Diagnostic tests

All study subjects had the following in vivo and/or in vitro tests.

SPTs

Patients in groups 1 and 2 had SPTs with the five allergens mentioned previously *(P. pratense, B. verrucosa, P. judaica, D. pteronyssinus,* and *F. domesticus)* with the Phazet lancet (Pharmacia).[9] This device was chosen for the study to reduce the influence of differences in the operator's SPT technique in different centers. The Phazet device, with preloaded allergen (no longer commercially available), was pressed at a 90-degree angle on the volar surface of the forearm and kept there for 1 second. Each allergen was tested in duplicate. A negative diluent control and a positive control (histamine 10 mg/ml) were included in each series of tests. The outline of the resulting wheal was marked with a pencil, transferred onto a piece of cellophane and then onto the patient's record, from which the diameters were later measured and the areas calculated at the coordinating center. The SPT response was considered positive when a wheal of at least 3 mm in diameter and 7 mm^2 in area was elicited, according to recommendations of the European Academy of Allergology and Clinical Immunology.[10]

In vitro tests

For patients in groups 1 and 2, IgE antibody was assayed with the CAP System only for the causal allergen that had been identified by history and SPT (patients with symptoms) or by SPT alone (patients without symptoms). For subjects in group 3, all five allergen specificities were assayed with the CAP System. Twenty milliliters of venous blood was drawn from each subject and centrifuged; and serum samples were collected and stored at −20° C until assays were performed. Blood samples were obtained during the same periods from the two groups of patients to avoid possible differences in specific IgE levels caused by differences in exposure to the allergens (such as the seasonal rise of IgE antibody in pollen-sensitive patients). In spite of this precaution, this factor cannot be entirely excluded because of the different geographic locations of the participating centers.

All of the CAP System assays were performed at the same time by the same laboratory (Institute of Clinical Biochemistry, University of Padua, Italy, Dr. Plebani) according to a technique previously reported in detail.[5] The immunoenzymatic method was used. Briefly, test sera were incubated with the solid phase, consisting of a flexible hydrophilic allergen carrier (polymer) encased in a capsule (the ImmunoCap [Pharmacia]). This carrier consists of a cyanogen bromide–activated cellulose derivative, which can bind at least 3 times more allergen than the corresponding paper disk used in RAST and up to 50 times more allergen than the amount adsorbed on a coated tube.[5] An anti-human IgE (polyclonal and monoclonal) antibody mixture labeled with β-galactosidase (generating fluorescence) was then added. This reagent has high immunoreactivity and low background, allowing a wider range of measurement compared with RAST.[5] Finally, the intensity of the resulting color was measured in a spectrophotometer. The entire procedure is automated. Results, expressed in kilounits per liter, were obtained by reference to a standard curve derived with serial dilutions of human IgE that was calibrated against the World Health Organization standard for IgE (standard WHO 75/502). One kilounit per liter corresponds to 2.4 ng of IgE per milliliter. A value greater than 0.35 kU/L is defined as a positive CAP System result.

TABLE I. Number of subjects with a positive CAP System assay result and total number of assays performed for each allergen (% positive*)

Allergen	Group 1	Group 2	Group 3
P. pratense	53/55 (96.3%)	35/39 (89.7%)	13/227 (5.7%)
B. verrucosa	41/42 (97.6%)	28/34 (80.8%)	5/206 (2.4%)
P. judaica	31/32 (96.8%)	22/22 (100%)	9/202 (4.4%)
D. pteronyssinus	125/134 (93.2%)	112/127 (88.1%)	21/243 (8.6%)
F. domesticus	4/4 (100%)	8/9 (88.8%)	5/230 (2.1%)

*A positive CAP System result is one greater than 0.35 kU/L

Data analysis

Differences in the levels of specific IgE among the three groups of subjects were analyzed by Student's *t* test. The cutoff levels of specific IgE discriminating patients with symptomatic allergy from those with asymptomatic allergy were determined by analysis of ROC curves.[11, 12] The analysis was performed by using the computer program Labroc-1 (written by C. Metz et al., University of Chicago), a modified version of the program "Rscore II,"[13] which establishes from the continuously distributed input data several operating points corresponding to a series of discriminator positions. Calculation and output of expected operating points are estimated on the fitted ROC curve together with asymmetric 95% confidence intervals for those points along the curve. The optimal cutoff was established by plotting the values of sensitivity (true positive results in patients with symptomatic allergy) and 1-specificity (1 − false-positive results in patients with asymptomatic allergy) obtained for each discriminator position and then determining the distance of the discriminator position from the ideal point represented by a sensitivity of 1 and a specificity of 1, that is, where 1-specificity equals 0. The optimal cutoff was obtained at the point of the curve at the minimal distance from the ideal point. For patients with symptomatic and asymptomatic allergy, the areas of SPT wheals were also analyzed by ROC curves. The diagnostic value, being the sum of the sensitivity and the specificity[14] and ranging from a minimum of 0 to a maximum of 200, of each discriminator position for specific IgE and SPT area was also calculated.

RESULTS

Table I shows the number of allergen-specific CAP System assays performed and the rate of positivity in each group of subjects. SPTs were not included because the percent positive is 100 in groups 1 and 2 (because the positivity of SPT was the inclusion criterion), and they were not performed in group 3 (screened by Phadiatop test).

The mean concentration of specific IgE for all five allergens combined was 31.71 ± 11.42 kU/L in patients with symptomatic allergy, 8.57 ± 5.63 kU/L in patients with asymptomatic allergy and 0.37 ± 0.06 kU/L in normal subjects. Both the difference between the specific IgE levels of patients with symptomatic and asymptomatic allergy and the difference between those of patients with symptomatic allergy and nonallergic subjects were highly significant ($p < 0.001$). Also, the difference between patients with asymptomatic allergy and nonallergic subjects was significant ($p < 0.01$).

The mean specific IgE values for the seasonal allergens *(P. pratense, B. verrucosa, P. judaica)* and the perennial allergens *(D. pteronyssinus* and *F. domesticus)* were then calculated for the two groups of patients with allergy. For the seasonal allergens the mean value for group 1 was 35.22 ± 10.81 kU/L, and for group 2 it was 8.83 ± 5.16 kU/L. For the perennial allergens the mean value for group 1 was 28.77 ± 12.02 kU/L, and for group 2 it was 8.39 ± 6.02 kU/L. In both cases the differences between the specific IgE levels for the two groups were significant ($p < 0.001$).

Figs. 1, 2, and 3 show ROC curves obtained by plotting sensitivity (true positive results in patients with symptomatic allergy) versus 1-specificity (1 − false-positive results in patients with asymptomatic allergy) from CAP System and SPT for the seasonal allergens plus *D. pteronyssinus* (*F. domesticus* was not included, because the number of patients was too small) (Fig. 1), for the seasonal allergens alone (Fig. 2), and for *D. pteronyssinus* (Fig. 3). The cutoff values were 11.7 kU/L for the IgE antibody and 32.2 mm^2 for the SPT for the four allergens, 10.7 and 32.4 for the seasonal allergens alone, and 8.4 kU/L and 31.2 mm^2 for *D. pteronyssinus.* The corresponding diagnostic values for the CAP System assays were 141.8 at 11.7 kU/L for the four allergens, 152 at 10.7 kU/L for seasonal allergens, and 134.4 at 8.4 kU/L for *D. pteronyssinus.* For SPT, the cutoff had a diagnostic value of 121.4 for the four allergens, 122.6 for seasonal allergens, and 121.9 for *D. pteronyssinus* (Table II).

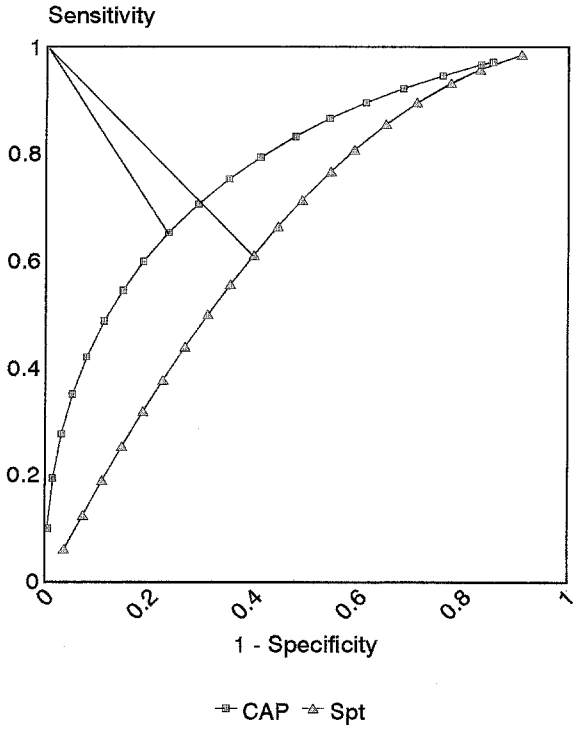


FIG. 1. ROC curve obtained with CAP System *(squares)* and SPT *(triangles)* by plotting sensitivity in patients with symptomatic versus 1-specificity in patients with asymptomatic allergy for all allergens.

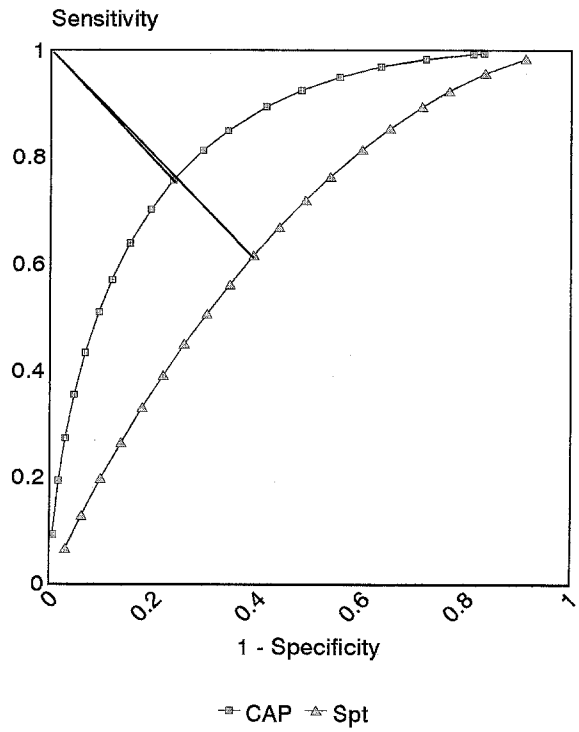


FIG. 2. ROC curve obtained with CAP System *(squares)* and SPT *(triangles)* by plotting sensitivity in patients with symptomatic allergy versus 1-specificity in patients with asymptomatic allergy for seasonal allergens.

DISCUSSION

In this study specific IgE antibodies were measured with a quantitative method in sera from two populations of patients with allergy, one with rhinoconjunctivitis and/or asthma caused by a common environmental allergen and another without symptoms of allergy to the same set of allergens, and from a large group of healthy, nonallergic control subjects. The mean antibody levels were significantly higher in the patients with symptomatic allergy compared with the patients with asymptomatic allergy, and a comparable difference was also observed between patients with symptomatic allergy and healthy control subjects. The mean antibody level in patients with asymptomatic allergy was also greater than the level in healthy control subjects, but to a lesser degree than it was for the patients with symptomatic allergy. These findings appear to be reliable because the three populations were defined according to very strict criteria, and confounding factors, such as when blood samples were taken, were also controlled.

A way to distinguish patients with symptomatic allergy from those with asymptomatic allergy is necessary in clinical practice because the simple presence of allergen-specific IgE antibodies to a particular environmental allergen does not mean that a patient has clinically significant symptoms when exposed to that allergen. It has long been recognized that a positive skin test response to an allergen in a patient who is free of symptoms cannot be regarded simply as a false-positive response because it is often possible to transfer the reaction passively to a nonallergic individual with the patient's serum.[15] The significance of allergen-specific IgE antibody in an individual without symptoms remains uncertain. It is, at least, evidence of previous sensitization,[16] and perhaps it is a marker of future allergic disease.[17-19]

The diagnosis of clinically relevant allergy depends, in the first instance, on a correlation between the clinical history and the results of skin tests. When a patient whose history is not definitive has positive skin test responses to multiple allergens, it is very difficult to distinguish clinically relevant allergens from those that are not relevant. One method that has been used in such cases is to challenge the target organ with the possibly relevant allergens.[20] Such tests are, however, risky and time-consuming. Moreover, their significance is not always different from that of the skin test, because the allergen doses commonly used in

challenge tests, which are usually much greater than those encountered during natural exposure, may elicit symptoms in subjects without apparent clinical sensitivity.

In regard to in vitro titration of allergen-specific IgE, the studies reported to date have considered the sensitivity, specificity, and efficiency of the available tests, comparing results obtained in allergic and nonallergic subjects. To our knowledge, there has not yet been a study aimed at discriminating patients with asymptomatic allergy from those with symptomatic allergy on the basis of their IgE antibody levels. In this study we used a quantitative assay, which has been reported to have very good diagnostic capabilities, to measure specific IgE.[5-7] In fact, this assay has greater sensitivity than the RAST, especially for allergens such as house dust mites and cat (which were included in our panel), with no decrease in specificity.[6, 21] We determined the optimal cutoff levels of the CAP System and the SPT from ROC curves. This approach is based on information theory[11] and has been used increasingly in medicine in recent years. It has also been used recently to determine cutoff values for SPTs,[22] for in vitro tests,[23] and for both of these.[24]

By ROC analysis, we found that the cutoff value between patients with symptomatic allergy and those with asymptomatic allergy was 10.7 kU/L for seasonal allergens and 8.4 kU/L for *D. pteronyssinus.* These cutoff values appear to be more useful than the cutoff values for SPT, because they have a greater diagnostic value (the sum of sensitivity and specificity): 152 for seasonal allergens and 134.4 for the perennial allergen with the CAP System and 122.6 and 121.9, respectively, for the SPT. This may seem surprising, because since the introduction of the RAST,[25] in vitro tests have always been found less sensitive than skin tests. This lower sensitivity was also confirmed in a recent study that compared three in vitro IgE antibody assays, including the CAP System, with skin tests.[24] Our results, however, cannot be compared with those of these other studies because we studied patients with positive skin test results with and without symptoms of allergy. Moreover, in our study the sensitivity of SPT (at least, for the first discriminator positions) was higher than that of the CAP System, but this was counterbalanced by the much lower specificity (i.e., greater number of false-positive results) of the SPT. The relative lack of specificity of the SPT accounts for the lower diagnostic value at the optimal cutoff value for the SPT.

The cutoff levels for the CAP System found in this study are proposed to be used as decisional levels in clinical practice when it is necessary to resolve the question of the relevance of a positive skin test response or, as is often the case, of multiple positive skin test responses, when there is no clear-cut clinical history of symptoms on exposure to the allergen or allergens that elicit positive responses. This is as yet only clear for aeroallergens. Other allergens, such as foods or Hymenoptera venoms, require further investigation. Furthermore, the decisional levels that we have proposed could be useful when a diagnosis of allergy is based only on in vitro test results, as in the so-called "remote practice of allergy." Such a practice, although criticized by the American Academy of Allergy and Immunology,[26] is being done increasingly and can lead to much unnecessary allergen immunotherapy.[4]

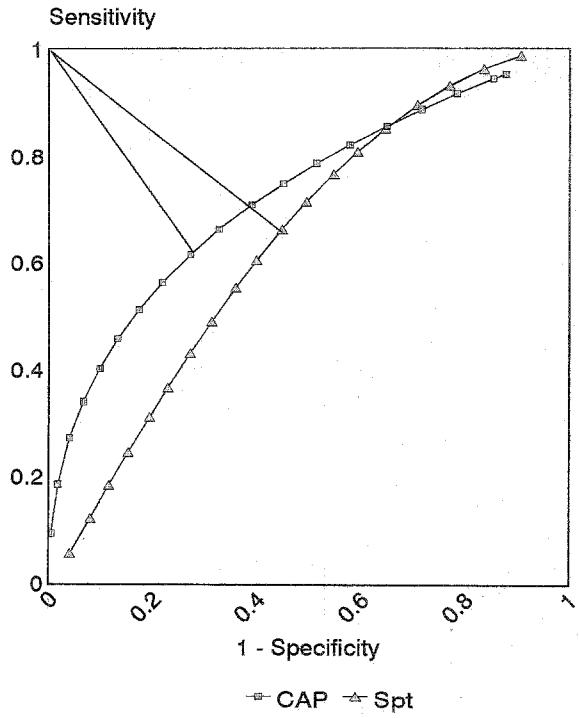


FIG. 3. ROC curve obtained with CAP System *(squares)* and SPT *(triangles)* by plotting sensitivity in patients with symptomatic allergy versus 1-specificity in patients with asymptomatic allergy for *D. pteronyssinus.*

In conclusion, we found a significant difference in the levels of allergen-specific IgE antibodies measured with the CAP System between two populations of subjects sensitized to common aeroallergens, and the levels were significantly lower in patients with asymptomatic allergy compared with those with symptomatic allergy. A cutoff level of 10.7 kU/L for seasonal allergens and 8.4 kU/L for

TABLE II. Cutoff values, sensitivity, specificity, and diagnostic values for CAP System and SPT

	Cutoff value		Sensitivity		Specificity		Diagnostic values	
	CAP (kU/L)	SPT (mm^2)	CAP (kU/L)	SPT (mm^2)	CAP (kU/L)	SPT (mm^2)	CAP (kU/L)	SPT (mm^2)
All allergens	11.7	32.2	65.2%	61.1%	76.6%	60.3%	141.8	121.4
Seasonal allergens	10.7	32.4	75.5%	61.6%	76.5%	61.0%	152.0	122.6
D. pteronyssinus	8.4	31.2	61.6%	66.3%	72.8%	55.6%	134.4	121.9

D. pteronyssinus was found to discriminate between these two groups of patients. The cutoff levels for SPT were, respectively, 32.4 mm^2 for seasonal allergens and 31.2 mm^2 for *D. pteronyssinus,* but their diagnostic value was lower than that of the in vitro assay.

We thank Dr. Gian Galeazzo Riario-Sforza for his skillful assistance in the analysis of data.

REFERENCES

1. Siraganian RP. Mechanism of IgE-mediated hypersensitivity. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW, eds. Allergy: principles and practice. 4th ed. St Louis: Mosby, 1993:105-34.
2. Ownby DR. Clinical significance of IgE. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW, eds. Allergy: principles and practice. 4th ed. St Louis: Mosby, 1993:1059-76.
3. Norman PS, Lichtenstein LM, Ishizaka K. Comparison of specific IgE antibodies, leukocyte sensitivity by histamine release, direct skin tests, and symptoms in hay fever. In: Goodfriend L, Sehon A, Orange R, eds. Mechanisms in allergy: reagin-mediated hypersensitivity. New York: Marcel Dekker, 1973:151-62.
4. Executive Committee of the American Academy of Allergy and Immunology. The use of in vitro tests for IgE antibody in the specific diagnosis of IgE-mediated disorders and in the formulation of allergen immunotherapy [Position statement]. J ALLERGY CLIN IMMUNOL 1992;90:263-7.
5. Ewan PW, Coote D. Evaluation of a capsulated hydrophilic carrier polymer (the immuno CAP) for measurement of specific IgE antibodies. Allergy 1990;45:22-9.
6. Bousquet J, Chanez P, Chanal I, Michel FB. Comparison between RAST and Pharmacia CAP System, a new automated specific IgE assay. J ALLERGY CLIN IMMUNOL 1990; 85:1039-43.
7. Pastorello EA, Incorvaia C, Pravettoni V, et al. A multicentric study on sensitivity and specificity of a new in vitro test for measurement of IgE antibodies. Ann Allergy 1991;67:365-70.
8. Eriksson NE. Allergy screening with Phadiatop^R and CAP Phadiatop^R in combination with a questionnaire in adults with asthma and rhinitis. Allergy 1990;45:285-92.
9. Holgersson M, Stahlenheim G, Dreborg S. The precision of skin prick test with Phazet™, the Osterballe needle and the bifurcated needle. Allergy 1985;40:64-6.
10. Dreborg S, ed. Skin tests used in type I allergy testing. Position paper of the European Academy of Allergology and Clinical Immunology. Allergy 1989;44(suppl 10): 27-8.
11. McNeil BJ, Hanley JA. Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. Med Decis Making 1984;4:137-50.
12. Gerhardt W, Keller H. Evaluation of test data from clinical studies. I. Terminology, graphic interpretation, diagnostic strategies and selection of sample groups. Scand J Clin Lab Invest 1986;46(suppl 181):5-42.
13. Swets JA, Pickett RM. Evaluation of diagnostic systems: methods from signal detection theory. New York: Academic Press, 1982.
14. Armitage P, Berry G. Statistical methods in medical research. 2nd ed. Oxford: Blackwell Scientific, 1987.
15. Lindblad JH, Farr RS. The incidence of positive intradermal reactions and the demonstration of skin sensitizing antibody to extracts of ragweed and house dust in humans without history of rhinitis or asthma. J Allergy 1961;32:392-401.
16. Yunginger JW. Diagnostic tests, total IgE levels and specific IgE antibody levels. In: Kaplan AP, ed. Allergy. New York: Churchill Livingstone, 1985:112-23.
17. Chambers VV, Glaser J. The incidence of subsequent ragweed pollinosis in symptom-free persons having positive reactions to ragweed-pollen extract. J Allergy 1958;29:249-57.
18. Hagy GW, Settipane GA. Risk factors for developing asthma and allergic rhinitis. J ALLERGY CLIN IMMUNOL 1976;58:330-6.
19. Horak F. Manifestation of allergic rhinitis in latent-sensitized patients. A prospective study. Arch Otorhinolaryngol 1985;242:224-39.
20. Naclerio RM, Norman PS, Fish JE. In vivo methods for the study of allergy. Mucosal tests, techniques, and interpretations. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW, eds. Allergy: principles and practice. 4th ed. St Louis: Mosby, 1993:595-627.
21. Pastorello EA, Incorvaia C, Pravettoni V, Marelli A, Farioli L, Ghezzi M. Clinical evaluation of CAP System and RAST in the measurement of specific IgE. Allergy 1992; 47:463-6.
22. Ollier S, Osman J, Hordle DA, Amin N, Overell B, Davies R. Skin prick test preparation of *Dermatophagoides pteronyssinus* for prediction of a positive response to provocation testing. Clin Exp Allergy 1989;19:457-62.
23. de Blay F, Zana H, Offner M, Verot A, Velten M, Pauli G. Receiver Operating Characteristic analysis: a useful method for a comparison of the clinical relevance of two in vitro IgE tests. J ALLERGY CLIN IMMUNOL 1993;92:255-63.
24. Williams PB, Dolen WK, Koepke JW, Selner JC. Comparison of skin testing and three *in vitro* assays for specific IgE

in the clinical evaluation of immediate hypersensitivity. Ann Allergy 1992;68:35-45.
25. Berg TLO, Johansson SGO. Allergy diagnosis with the radioallergsorbent test: a comparison with the results of skin and provocation tests in an unselected group of children with asthma and hay fever. J ALLERGY CLIN IMMUNOL 1974;54:209-21.
26. American Academy of Allergy and Immunology. The remote practice of allergy [Position statement]. J ALLERGY CLIN IMMUNOL 1986;77:651-2.

APPENDIX
Centers participating in the study

Ospedale Borgo Trento, Verona (L. Andri)
Ospedale Regionale Umberto I, Ancona (F. Bonifazi)
Ospedale Civile, Brescia (R. Cattaneo)
Ospedale Ascoli Tomaselli, Catania (N. Crimi)
Ospedale Civile, Cagliari (G. S. Del Giacco)
Ospedale Civile, Novara (M. Galimberti)
Policlinico Gazzi, Messina (G. Girbino)
Ospedale Civile, Palermo (A. Lococo)
Ospedale G. Mazzoni, Ascoli Piceno (G. Nardi)
Ospedale S. Martino, Genova (A. C. Negrini)
Ospedale Civile, Pordenone (G. Santini)
Ospedale Civile, Piacenza (E. Savi)
Dipartimento di Medicina Interna, L'Aquila (G. Tonietti)
Ospedale Borgo Roma, Verona (G. Tridente)
Ospedale A. Sclavo, Siena (M. Vagliasindi)
Nuovo Ospedale S. Giovanni di Dio, Firenze (R. Zerboni)

Coordinating Centers

Allergy Center, First Department of Internal Medicine, University of Milan
Bizzozero Division of General Medicine, Niguarda Cà Granda Hospital, Milan
Andrea Cesalpino Foundation, First Department of Internal Medicine, University "La Sapienza," Rome
Allergy and Clinical Immunology Center, Scientific Department of Internal Medicine, University of Genoa
Department of Allergy and Clinical Immunology, University of Florence
Department of Allergy and Clinical Immunology, University of Bari

Malaria Journal

BioMed Central

Research

Open Access

Total and functional parasite specific IgE responses in *Plasmodium falciparum*-infected patients exhibiting different clinical status

Joana Duarte[1], Prakash Deshpande[2], Vincent Guiyedi[3,4], Salah Mécheri[5], Constantin Fesel[1], Pierre-André Cazenave[3], Gyan C Mishra[2], Maryvonne Kombila[4] and Sylviane Pied*[1,3]

Address: [1]Instituto Gulbenkian de Ciencia, LEA CNRS-IGC, Oeiras, Portugal, [2]National Centre for Cell Sciences, Pune, India, [3]Unité d'Immunophysiopathologie Infectieuse, Institut Pasteur, 25 rue du Docteur Roux, 75 724, Paris Cedex 15, France, [4]Département de Parasitologie-Mycologie-Médecine Tropicale, Faculté de Médecine de Libreville, Gabon and [5]Unité des Réponses Immunes Précoces aux Parasites, Institut Pasteur Paris, France

Email: Joana Duarte - jduarte@igc.gulbenkian.pt; Prakash Deshpande - pdeshpande@nccs.in; Vincent Guiyedi - guidivin@pasteur.fr; Salah Mécheri - mecheri@pasteur.fr; Constantin Fesel - cfesel@igc.gulbenkian.pt; Pierre-André Cazenave - cazenave@pasteur.fr; Gyan C Mishra - gcishra@nccs.in; Maryvonne Kombila - valentine_favry@yahoo.fr; Sylviane Pied* - spied@pasteur.fr

* Corresponding author

Published: 04 January 2007

Malaria Journal 2007, **6**:1 doi:10.1186/1475-2875-6-1

Received: 25 October 2006
Accepted: 04 January 2007

This article is available from: http://www.malariajournal.com/content/6/1/1

Abstract

Background: There is an increase of serum levels of IgE during *Plasmodium falciparum* infections in individuals living in endemic areas. These IgEs either protect against malaria or increase malaria pathogenesis. To get an insight into the exact role played by IgE in the outcome of *P. falciparum* infection, total IgE levels and functional anti-parasite IgE response were studied in children and adults, from two different endemic areas Gabon and India, exhibiting either uncomplicated malaria, severe non cerebral malaria or cerebral malaria, in comparison with control individuals.

Methodology and results: Blood samples were collected from controls and *P. falciparum*-infected patients before treatment on the day of hospitalization (day 0) in India and, in addition, on days 7 and 30 after treatment in Gabon. Total IgE levels were determined by ELISA and functional *P. falciparum*-specific IgE were estimated using a mast cell line RBL-2H3 transfected with a human Fcε RI α-chain that triggers degranulation upon human IgE cross-linking. Mann Whitney and Kruskall Wallis tests were used to compare groups and the Spearman test was used for correlations.

Total IgE levels were confirmed to increase upon infection and differ with level of transmission and age but were not directly related to the disease phenotype. All studied groups exhibited functional parasite-specific IgEs able to induce mast cell degranulation *in vitro* in the presence of *P. falciparum* antigens. Plasma IgE levels correlated with those of IL-10 in uncomplicated malaria patients from Gabon. In Indian patients, plasma IFN-γ , TNF and IL-10 levels were significantly correlated with IgE concentrations in all groups.

Conclusion: Circulating levels of total IgE do not appear to correlate with protection or pathology, or with anti-inflammatory cytokine pattern bias during malaria. On the contrary, the *P. falciparum*-specific IgE response seems to contribute to the control of parasites, since functional activity was higher in asymptomatic and uncomplicated malaria patients than in severe or cerebral malaria groups.

Background

Malaria is a complex disease that kills between one and two million people every year. Most of those affected are children under five years of age, non-immune individuals and pregnant women [1]. The principal cause of death is infection by *Plasmodium falciparum* due to its ability to induce severe complications such as severe anaemia and/or cerebral malaria (CM) often associated with hypoglycaemia [2-4]. The physiopathology of malaria cannot be represented by a single scheme. For example, patients who develop CM present a range of acute neurological manifestations and the disease is characterized by a diffuse encephalopathy, altered levels of consciousness, deep coma and seizure leading to death. Even though during the last few years a lot of information has become available from clinical and experimental studies, the causes of CM remain to be determined. The clinical outcome of a *P. falciparum* infection depends on the genetic factors of the host and parasite, and also on host immune responses. Antibodies and T cells are among the immune factors thought to play a role in mediating protection and also pathology [2-5].

P. falciparum infection increases the serum levels of IgM and IgG antibodies but also IgE in individuals living in endemic areas [6-12]. IgEs may protect against or participate in malaria pathogenesis. The association of high anti-*P. falciparum* IgE levels with a reduced risk of developing clinical malaria suggests the involvement of IgE in protection [13,14]. The observation that circulating levels of IgE most often correlate with severe rather than uncomplicated disease suggests a pathogenic role of IgE [8,10-12], and the positive correlation between the levels of IgE/IgE immune complexes and the levels of TNF in CM patients provides supporting evidence [8,10-12]. The exact role played by IgE in malaria is still unclear.

IgE is an immunoglobulin isotype that only exists in mammals. It is present at very low concentrations in the serum of normal individuals, at levels ranging from 10 to 300 ng/ml [9]. Its functional effect has been shown to depends on Fc receptors expressed on mast cells and basophils both in mice and humans, as well as on eosinophils, monocytes/macrophages and platelets in humans [9]. IgEs positively regulate both of their receptors: the high affinity receptor (Fcε RI) and the low affinity receptor (Fcε RII or CD23) [15]. The Fcε RI is expressed only on mast cells and/or basophils in both mice and humans [9,16]. The binding of IgE to the high affinity receptor on the mast cell membrane and its subsequent aggregation with antigens results in degranulation and the release of mediators that further aggravate an ongoing allergic process [17]. On basophils, the cross-linking of Fcε RI-bound IgE rapidly induces the release of IL-4 and IL-13 [16], among other inflammatory mediators. The low affinity receptor (Fcε RII) is the second major and widely distributed IgE receptor. It is also known as CD23 and is constitutively expressed on B cells and is induced by IL-4 on macrophages, some T cells, human eosinophils and platelets [9,16]. The cross-linking of CD23 on macrophages or on other CD23-bearing effector cells by IgE-containing immune complexes is thought to play a pathogenic role in malaria via TNF-mediated pathways [16].

This study aimed to evaluate the total and functional *P. falciparum*-specific IgE responses, the association of these responses with plasma cytokine patterns and the phenotype of the disease in endemic controls and infected patients with different clinical forms of malaria. The infected patients originated from a low endemic area in India and a high endemic area in Gabon.

Materials and methods

Study population

Patients from Gabon

All the patients included in this study were children aged between 0.1 and 6 years (mean age = 2.6 years) recruited between 1996 and 2000 at the Owendo Pediatric Hospital (OPH) and the Libreville Hospital Center (LHC) in Gabon (see Table 1). Informed parental consent had been obtained. Gabon has an equatorial climate that is hot and humid, with an endemic malaria transmission. The study design was approved by the local health office ethics committee. The patients were distributed into different groups according to World Health Organization (WHO) guidelines for the definition of uncomplicated and severe malaria [18]. A cohort of 135 *P. falciparum*-infected children was constituted and divided into three groups according to disease severity [[6], 67] 50patients with uncomplicated malaria (UM), 29 with severe non-cerebral malaria (SM) developing severe anaemia (haemoglobin level < 5 g/dl), or hypoglycaemia (glycaemia < 2.2 mmol/ml), and 17 with severe cerebral malaria (CM) with a Blantyre Coma Score < 2, or three convulsive episodes during 24 hours before admission with post-critical comatose > 15 minutes]. Two control groups were recruited: an uninfected group, also called endemic control (EC) group, comprising 17 children with *P. falciparum*-negative thin blood smear, and asymptomatic infected group (AI) comprising 22 children with no clinical manifestation of malaria but a *P. falciparum*-positive thin blood smear.

Patients from India

Malaria patients were recruited in the village of Gondia, an endemic region in the north-east of the Maharashtra State of India. The village is surrounded by forest. Gondia has been known as an endemic area for at least the last 20 years. *P. falciparum* appeared in Gondia over the last 10 years. It is transmitted during the rainy season in June,

Table 1: Characteristics of both studied cohorts: Gabon and India. Clinical group description, according to the number of patients, age, sex and parasitaemia.

	Clinical Groups	Staff	**Age-mean** (min-max)	**Sex** (male-female)	**Parasitemia** (%)
Gabon	**EC**	17	2,7 (0,5–5)	10-7	-
	AI	22	2,9 (0,1–5)	12-10	0.22 (0.01–1.2)
	UM	50	3 (0,5–5)	22–28	6.88 (0.05–48.6)
	SM	29	1,8 (0,2–4,5)	18-11	5.91 (0.08–31)
	CM	17	2,4 (0,5–5)	12-5	10.42 (0.15–64)
	Total	145	2,6 (0,1–5)	74-61	
Índia	**NEC**	9	32,7 (25–63)	8-1	-
	EC	14	27,3 (23–37)	13-1	-
	UM	31	30,8 (4–70)	18-13	1,24 (0,71–2,18)
	SM	13	30,4 (8–65)	10-3	1,11 (0,38–3,21)
	CM	26	40,4 (9–72)	16-10	2,04 (1,07–3,87)
	ExCM	5	19,36 (7–51)	0–5	-
	Total	93	32,3 (4–72)	65-28	1,50 (1,02–2,20)

NEC- non endemic control, EC- endemic control, AI- asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria, ExCM – Ex-cerebral malaria. Note: sex couldn't be determined for 1/18, 11/61 and 4/33 patients from EC, UM and SM, respectively.

peaks in the winter season (November, December and January) and becomes rare as summer approaches (March, April and May). The studied groups consisted of 98 patients from four to 72 years of age, being predominantly adults (see Table 1). Six cohorts were constituted according to WHO criteria for uncomplicated and severe malaria: two control groups of uninfected individuals from non-endemic (NEC) and endemic regions (EC) comprising nine and 14 patients respectively; three groups of infected patients, with 31 developing uncomplicated malaria (UM), 13 developing severe non-cerebral malaria (SM) and 26 developing cerebral malaria (CM); and one group of five patients that had recovered from CM (ex-cerebral malaria patients, Ex-CM). Between eight and 10% of the CM malaria cases died. UM cases were treated as outpatients. SM patients were admitted to hospital fully conscious and could respond well verbally to doctors' questions. CM cases were in coma. Drug treatment was paracetamol, quinine and arteether (E-mal®). Samples were collected after obtaining the consent of the patients, or of their families. Blood samples from endemic controls were collected from the relatives of malaria patients (brothers/sisters/parents) with their consent. These controls had not suffered from malaria during the previous two years. Non-endemic blood samples were collected from individuals who had not suffered from malaria during the previous five years.

Blood sample collection and parasite assessment

Venous blood was collected on EDTA in sterile vacutainers from each patient on the day of hospitalization (day 0, before any treatment), and seven (day 7) and thirty days later (day 30). Plasma was obtained by centrifuging the blood samples at 5000 rpm for 15 min. Plasma samples were stored at -80°C until use.

Parasitaemia was assessed by counting asexual forms of *P. falciparum* on thin blood smears under a light microscope after Giemsa staining. Parasitaemia was expressed as the mean percentage of infected red blood cells.

Culture of malaria parasites

Erythrocytic stages of the *P. falciparum* malaria parasite line *FAN 5HS* (source: NCCS, Pune, India) and 3D7 were cultured using candle jar dessicators as previously described [19]. The culture medium was RPMI 1640 (Gibco-BRL), supplemented with 0.5% AlbuMix (Gibco BRL). The cultures were maintained in six-well or 24-well tissue culture plates (NUNC). Parasitaemia was 5% at the start of culture and reached 25% after six days. Culture medium and fresh RBCs were added every other day.

Preparation of parasite extracts

Parasite soluble antigen was prepared from synchronous cultures containing more than 20% mature trophozoites; more than 6% rings and more than 5% schizonts were used. The cultures were pooled and centrifuged at 3,000 rpm at 4°C, and the pRBC pellet was kept and the supernatant discarded. The pRBC pellet was suspended in 10 ml sterile PBS 1 × (0.15 M, pH7.2) and then centrifuged. The parasitized red blood cell (pRBC) pellet was washed five times and then lysed by adding 15 ml of 0.1% saponin. The saponin treatment frees the parasites from the infected RBCs. This was centrifuged at 6,000 rpm for 30 min at 4°C. The supernatant was discarded and the parasite pellet was washed five or six times with sterile

cold PBS. The parasite pellet was resuspended in 1 ml protein isolation buffer containing a cocktail of protease inhibitors. This was briefly sonicated and the tube was kept at 4°C for between four and five hours. The contents of the tube were agitated by cyclo-mixing and then centrifuged at 6,000 rpm for 30 min at 4°C. The clean supernatant was collected in a separate tube and the pellet was discarded. The contents were sterilized by passing through 0.22 μm-pore filters. Aliquots of the antigen were frozen at -70°C until use. Parasite proteins were quantified by the Bradford method. The concentration of the parasite line FAN 5HS and 3D7 were 1.2 and 2.6 mg/ml, respectively.

Normal RBC extracts

Normal red blood cell (RBC) extract was prepared from the same batch of RBCs used for culturing the parasites, and followed the same procedure as previously described for pRBCs. Briefly, the RBCs were washed with PBS and the buffy coat was removed. After centrifugation, the RBC pellet was suspended in 1 ml protein isolation buffer containing a cocktail of protease inhibitors. This was briefly sonicated and the tube was kept at 4°C for between four and five hours. The contents of the tube were agitated by cyclo-mixing and then centrifuged at 6000 rpm for 30 min at 4°C. The clean supernatant was collected in a separate tube and the pellet discarded. The protein contents were estimated using a protein determination kit (BCATM protein assay Kit, Pierce, France).

Total IgE levels

An ELISA method was used to detect total IgE plasma levels in samples corresponding to day 0, day 7 and day 30. ELISA plates (96 microwell plates, reacti-bind 96 EIA Plate 100/PKG, Pierce) were coated with 50 μl/well of purified sheep polyclonal anti-human IgE solution at 5 μg/ml (The Binding Site, Birmingham UK) by incubation overnight at 4°C. The plasma samples were diluted 1:5 and incubated for two hours at 37°C. Bound IgE was detected using a peroxydase-conjugated polyclonal anti-human-IgE (The Binding Site, Birmingham UK). Binding was revealed using the OPD substrate (Sigma) and the product was quantified from the optical density (OD) at 450 nm. Serial dilutions ranging from 2 μg/ml to 0.0019 μg/ml of IgE solution (human monoclonal IgE provided by Dr Thierry Batard – Stallergenes, Anthony, France) gave the standard curve. The median of each optical density value was fitted into the sigmoidal standard curve using a specific ELISA programme running in Igor version 3.16 (Wavemetrics, Lake Oswego, OR).

IgE functional assay

A new rat mast cell line RBL-2H3 transfected with a human Fcε RI α-chain that triggers degranulation upon human IgE cross-linking was used [20]. Cells were maintained in Dulbecco medium (Gibco BRL, Eragny, France) containing 10% foetal bovine serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin (GIBCO BRL, France). Cells were expanded by incubation at 37°C for three to four days in complete Dulbecco medium supplemented with G418 (GIBCO BRL, France).

β-Hexosaminidase is known as a component of the basophil and the mast cell specific granule, and is released during degranulation of these cells [21]. Degranulation was monitored after antigen stimulation by measuring the level of released β-hexosaminidase. Fcε RI α-chain RBL-2H3 transfected rat mast cell line cultures (5×10^5 cells per well) were incubated with the different serum samples at a non-cytotoxic dilution (previously determined) for 48 hours at 37°C in the absence of the G418 antibiotic. The upregulated receptors were saturated by incubation at 4°C for 30 minutes with the same samples diluted 1:10. The cells were then washed with PBS 1X, centrifuged and resuspended in 1 ml Tyrode buffer before being centrifuged again. Finally, the cell pellet was resuspended in 450 μl of D2O (50%) and Tyrode buffer (50%) solution and each culture sample was distributed to 10 ELISA plate wells. Different controls were carried out for each sample. Control cells on lane 1 and 2 were subjected to Triton disruption (Triton 5%) and represented 100% enzyme release. Cells on lanes 3 and 4 were incubated with 50 μl of complemented Dulbecco medium without serum and represented the background enzyme release. Lanes 5 and 6, 7 and 8, 9 and 10 were incubated with 50 μl of different duplicated concentrations of parasite extract (1,000, 100 and 10 ng/ml) for 30 minutes at 37°C. After centrifugation of each well sample, 50 μl of each supernatant was collected and incubated with 50 μl of PNAG substrate solution for 90 minutes at 37°C. The level of released β-hexosaminidase was estimated from the OD at 405 nm using a spectrophotometer. All results are expressed as the percentage of total β-hexosaminidase in the cells after correcting for spontaneous release in unstimulated cultures, calculated as following: (experimental β-hexosaminidase – background β-hexosaminidase)/(total β-hexosaminidase – background β-hexosaminidase) × 100.

Flow cytofluorometry analysis

FACS analysis was performed after incubating RBL-2H3-D12.8 cells with several dilutions of serum samples to follow the induction of the high affinity receptor (Fcε RI) expression after stimulation by IgEs in the patient's sera. Cells were incubated for 48 hours at 37°C with the different serum samples optimally diluted to avoid cytotoxicity. A saturation step with the same sera diluted 1:10 was done by incubation at 4°C for 30 minutes. Cells were washed with PBS 1X and incubated with FITC-labelled anti-IgE (Tebu, Le Perray en Yvelines, France) (1/100) for 30 minutes. Cells were washed again, centrifuged, resuspended in

PBS 1X and analysed by cytofluorometry using Cellquest software (Beckton Dickinson, USA). 10,000 cells were acquired per tube.

Cytokine levels

The levels of cytokines in the plasma (IL-4, TNF, INF-γ, and IL-10) were estimated by Opti-ELISA kits (Pharmingen, San Diego, CA.USA) used following the manufacturer's instructions.

Statistics

Due to a non-normal distribution of the scores in each group, non-parametric tests were performed, using the median to compare the different clinical groups. The Mann Whitney test was used for comparisons between two groups and the Kruskall Wallis test to compare three or more groups. Spearman's correlation was used to check for correlations between parameters. P values less than 0.05 were considered as significant. Chi-squared test was used to compare qualitative variables.

Results

Serum total IgE levels in groups of P. falciparum *infected patients with different clinical phenotypes*

Total IgE levels were analysed in endemic controls and in cohorts of *P. falciparum*-infected patients with different clinical forms of malaria, ranging from asymptomatic to cerebral disease, from Gabonese and Indian endemic areas to study the association between the IgE response and disease severity. Total IgE levels were measured by ELISA in individual sera before drug administration (corresponding to day 0) and determined the general distribution in the studied populations from Gabon (Figure 1A) and India (Figure 1B). Total IgE concentrations were found to be much higher in patients from India (mainly adults) than in patients from Gabon (children). In both populations independent of the different levels of IgE in each population, the median IgE levels within each clinical group tended to increase upon infection (mainly in UM and SM groups), although the difference between the groups was only significant in the Indian population (Kruskall Wallis, p = 0.0005). As only Indian patients showed a significant difference, the Mann Whitney test was used to compare the different groups in this population only. There was a significant increase in IgE levels in the EC group compared to the NEC group (p = 0.042). The most significant increase in IgE levels (versus the EC group) occurred in the UM patients (p = 0.015) and in the SM patients (p = 0.013). No significant difference between the EC group and the CM and Ex-CM groups was observed.

A range of values of IgE levels was defined enabling the analysis of the frequency of normal, moderate and high IgE levels in each clinical group of patients. The so-called normal values were adjusted to the studied population because the Gabonese and Indian groups had different plasma total IgE ranges. Therefore, the normal value (N) was defined by the median IgE levels in the endemic controls of each study population. Consequently, all values between N and 2N were considered as low/moderate IgE levels and those between 2N and 3N as moderate/high IgE levels, with the highest levels being above 3N (Figures 1C and 1D). Even in Gabonese patients, for whom the increase of IgE in the disease groups was not significant, a higher percentage of patients with clinical disease had higher IgE levels than controls and asymptomatic patients. These differences were more marked in the UM, SM and CM Indian patients (Figure 1D). In the Indian population, the NEC group did not have moderate/high IgE levels, although a high percentage of patients exhibited normal IgE levels (Figure 1D). Also, no significant change was detected in IgE levels over time in the UM, SM and CM groups of the Gabonese cohorts when tested seven days and 30 days after treatment (Table 2). No significant association of malaria and IgE levels with sex in the two studied populations. However, a significant increase in IgE levels with age (p = 0.00034) was observed in the Gabonese subjects (Figure 2A) but not in the Indian subjects.

The correlation between IgE levels and the parasite load was tested. Although the general trend was different in the Indian and Gabonese population, there was no significant correlation between IgE concentration and parasite load for all groups together. In Gabonese cohorts, a negative correlation for all groups was observed, except for the UM patients where the correlation showed a positive tendency. In the Indian cohorts, a positive correlation was observed between IgE levels and parasite load, mainly in the UM and SM groups (Figure 2B).

Functional parasite specific IgE response in P. falciparum *infected patients*

Previous studies have used ELISA to quantify specific IgE present in the serum [7,8,10,11]. The functionality of specific IgEs present in the serum was studied by evaluating the ability of these IgEs to induce mast cell degranulation in the presence of the parasite antigen. A rat mast cell line transfected with the human α-chain of Fcε RI was used [20]. Human Fcε RI expression was induced after incubation with all serum samples at non-cytotoxic dilutions. FACS was used to detect the presence of FcåRI receptors on the mast cells surface induced by IgE present in serum samples. Although the fluorescence intensity revealing human Fcε RI expression by the mast cells varied between patient samples, IgE receptors were upregulated in all the samples tested. No correlation between total IgE levels in the serum and the up-regulation of mast cell receptors was found. There was no significant correlation between IgE

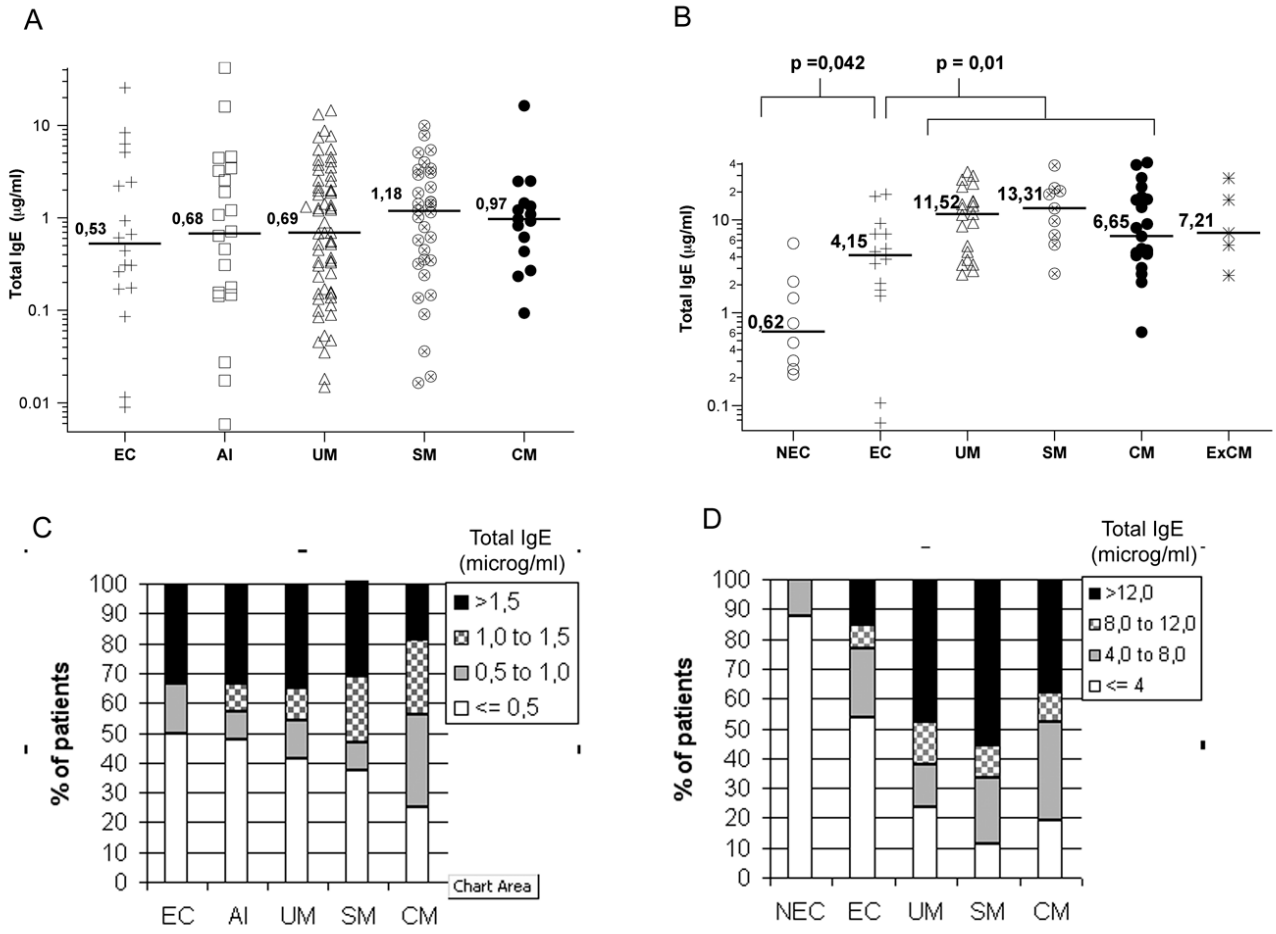


Figure 1

Distribution of total IgE levels per clinical group in both studied populations: Gabon and India. **A.** Total IgE levels (µg/ml) per clinical group in Gabonese patients (non-significant Kruskall Wallis test). **B**. Total IgE levels (µg/ml) in Indian patients (significant Kruskall Wallis test, p = 0.0005). **C.** Percentage of patients with defined IgE levels per group in the Gabonese population (normal levels (N) lower than or equal to 0.500 µg/ml, moderate levels (N to 2N), from 0.501 to 1.000 µg/ml, high levels (2N to 3N), from 1,000 to 1,500 µg/ml, very high (>3N) greater than 1,500 µg/ml). **D.** Percentage of patients with defined levels of IgE per group in the Indian population (normal levels (N) lower than or equal to 4,000 µg/ml, moderate levels (N to 2N), from 4,000 to 8,000 µg/ml, high levels (2N to 3N), from 8,000 to 12,000 µg/ml, very high (>3N), greater than 12,000 µg/ml). Legend: EC – endemic control, AI – Asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria, CM – cerebral malaria, NEC – non-endemic control, ExCM – ex-cerebral malaria.

levels and receptor upregulation and the level of mast cell degranulation. Sensitized cells were then incubated with different concentrations of a *P. falciparum* blood-stage antigen extract. Mast cell degranulation was measured by quantifying the release of β-hexosaminidase. Control cells not exposed to any serum gave a maximum mast cell degranulation of 3%. Therefore, serum samples giving an enzyme release greater than 5% in the presence of at least one of the antigen concentrations were considered positive for functional IgE. In the Gabonese cohorts (Figure 3A), there were functional *P. falciparum* IgE in all clinical groups. However, the percentage of patients with functional specific anti-parasite IgE was higher in asymptomatic and uncomplicated malaria patients than in other groups. Also, the percentage of patients displaying parasite-specific IgE was lower in the group exhibiting severe disease. The distribution of patients per group releasing between 5 and 10%, 10 and 30% and above 30% β-hexosaminidase induced by specific anti-parasite IgE revealed that one patient in CM group had a degranulation level above 30%, being the highest induced response among all the tested individuals. The same assay was carried out on

Table 2: Day 0, day 7 and day 30 median IgE levels per clinical group in the Gabonese population.

Total IgE (μg/ml)	EC (min-max)	AI (min-max)	UM (min-max)	SM (min-max)	CM (min-max)
Day 0	0,525 (0,009–25,47)	0,677 (0–41,96)	0,690 (0,015–13,173)	1,132 (0,016–9,915)	0,922 (0,093–16,34)
Day 7	0,582 (0,0144–5,644)	0,448 (0,0192–3,188)	0,516 (0,011–5,228)	0,836 (0,016–5,689)	8,033 (8,033–8,033)
Day 30	1,026 (0,044–2,628)	0,977 (0,056–7,446)	0,151 (0,052–3,925)	0,662 (0,011–6,315)	------

EC – endemic control, AI – asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria.

the Indian population. Although there was no significant difference between groups, the percentage of patients having functional IgE recognizing the parasite extract was slightly higher in EC and UM groups than in SM and CM groups (Figure 3B). All positive patients had an enzyme release of between 10 and 30%. No significant correlation was found between *P. falciparum*-specific IgE-induced mast cell degranulation levels and sex, age and parasitaemia.

Relationship between total and specific serum IgE and cytokine profiles

IgE production is influenced by cytokines produced by activated T cells. These cytokines are also involved in pathophysiological mechanisms associated with severe malaria [4,22,23]. Therefore, the relationship between the cytokine profile, the IgE levels and the clinical manifestation was investigated. IFN-γ, TNF and IL-10 levels were measured in the sera of the Indian and Gabonese patients. IL-4 levels were measured only in Indian patients as it was not different between the Gabonese groups. IFNγ concentrations were highest in the Gabonese AI and CM groups (Table 3). This cytokine is significantly higher in the AI and CM groups than in EC ($p = 0.02$ and $p = 0.009$ respectively). The plasma TNF concentration was similar in the severe SM and CM groups and in disease-free EC and AI groups. TNF levels were clearly higher in SM and CM groups than in the UM group ($p < 0.001$). Surprisingly, EC and AI also exhibited higher TNF levels when compared to UM group. No association between IFN-γ or TNF and IgE levels were found. However, a significant positive correlation was found between the concentration of total IgE and IL-10 in the UM group ($p = 0.02$) and a significant negative correlation in the AI group ($p = 0.02$) (Figure 4). The median levels of the different cytokines in the plasma of Indian patients are given in Table 4. IL-10 and TNF levels were higher in CM patients than in controls and other *P. falciparum*-infected patients. The plasma concentrations of these cytokines were moderate in cured CM patients (ExCM). Their levels of IL-10 and TNF were slightly higher in endemic controls than in non-endemic controls. Levels of IFN- were lower in the CM group than in AI group. No

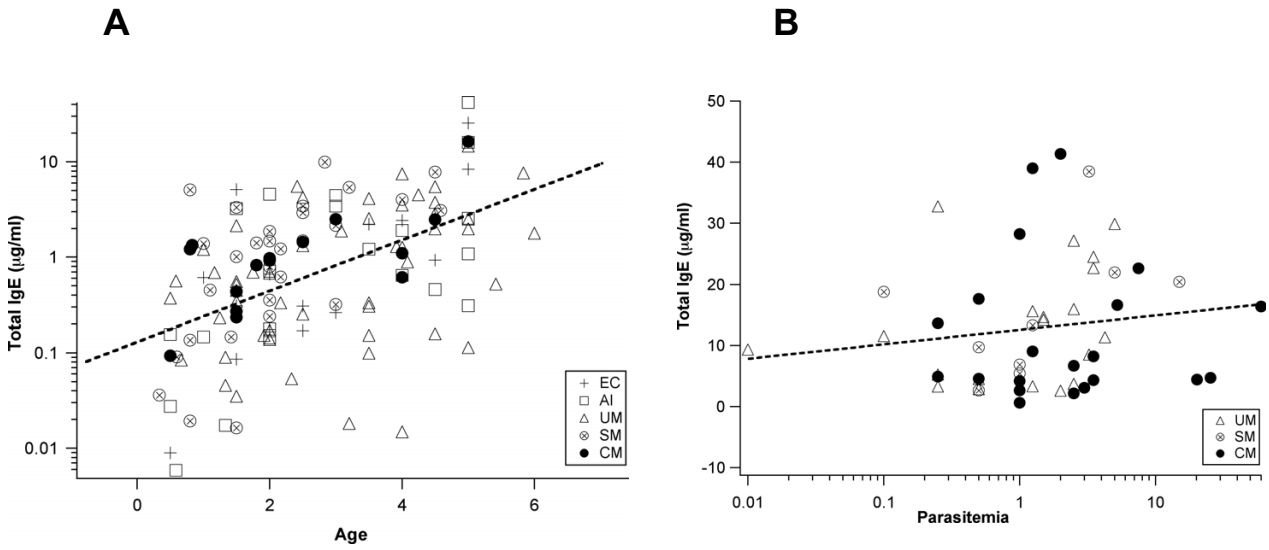


Figure 2
A. Total IgE correlation with age in the Gabonese population (significant spearman correlation, p = 1.0 × 10-9). **B.** Total IgE correlation with parasitaemia in the Indian population. Significant Spearman correlation (p = 0,0001).

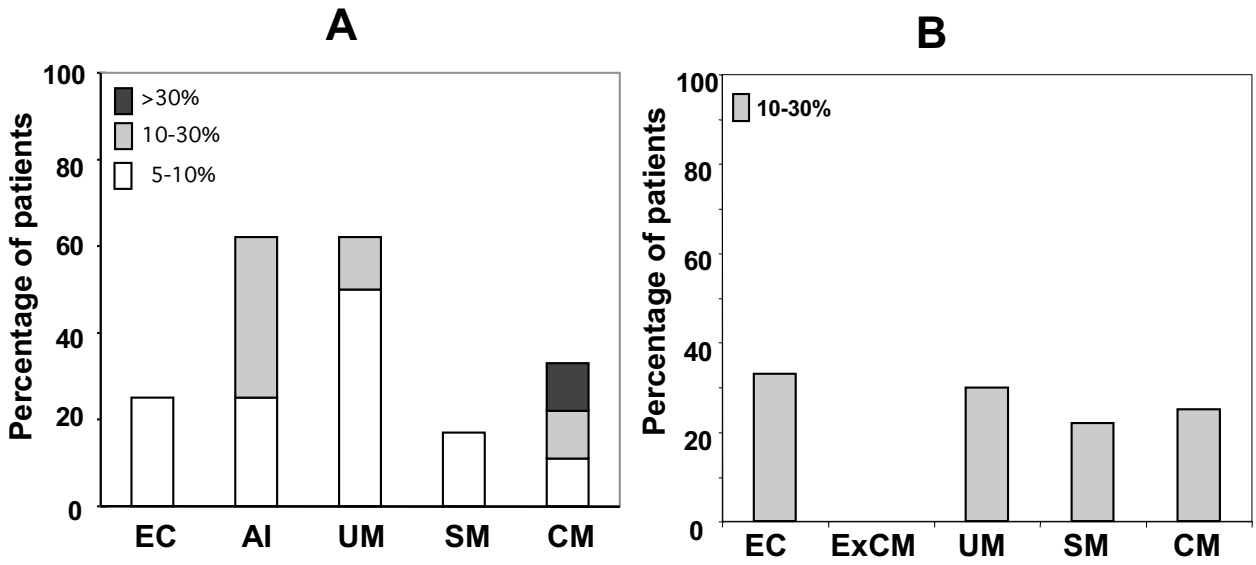


Figure 3
Percentage of patients with positive functional IgE against parasite antigen in the Gabonese and Indian populations. **A.** Distribution of patients with positive anti-parasite functional IgE, exhibiting different intensities of enzyme release per clinical group in the Gabonese population (low enzyme release, from 5 to 10%; moderate enzyme release, from 10 to 30%; and high enzyme release, greater than than 30%). **B.** Distribution of patients with positive anti-parasite functional IgE in the Indian population.

difference was found for the levels of IFN-γ between the uncomplicated and severe disease. Although, there was a significant correlation between IgE levels and IFN-γ (Figure 5A), TNF (Figure 5B) and IL-10 (Figure 5C) levels when looking at all the groups combined. Most diseased groups had high cytokine levels, whereas control groups had lower levels (Figure 5A, B and 5C).

Discussion

The main feature of this study is the comparison of total and functional *P. falciparum*-specific IgE responses in two populations of low and high malaria transmission levels, India and Gabon respectively and their relationship with disease severity. Several clinical groups were compared: endemic non-infected controls, asymptomatics and different clinical manifestations including uncomplicated, severe non-cerebral and cerebral malaria.

Indian and Gabonese individuals exhibited a different range of plasma levels of circulating IgE These cohorts had a different age range, with the Gabonese groups being children from 0 to 6 years of age, whereas Indian patients had a mean age of 30 years. In the two populations, irrespective of its concentration and also consistent with previously published data, the IgE distribution tended to increase upon parasite stimulation [8,10,11]. In the Indian population, circulating IgE levels were seven times higher in endemic controls than in non-endemic controls. This suggests that exposure to the parasite strongly influences the production of IgE, although this difference may also be due to other endemic factors [7]. Nevertheless, it was reported that IgE levels were greatest in patients developing severe disease than in CM group (Figure 1A and 1B). When considering the percentage of patients that produce high levels of IgE per group, it was shown that IgE

Table 3: Cytokine distribution in the Gabonese population: median TNF, IFN-γ and IL-10 levels per clinical group.

	EC (min-max)	AI (min-max)	UM (min-max)	SM (min-max)	CM (min-max)
TNF (pg/ml)	41 (8–94)	36 (0–395)	8 (0–440)	175 (1–442)	209 (0–1520)
IFN-γ (pg/ml)	3,2 (0–8)	9,5 (0–31)	5 (0–395)	4 (0–201)	6,2 (2,5–9)
IL-10 (pg/ml)	14 (0–83)	102 (0–317)	134 (0–1380)	339 (0–5200)	95 (0–2300)

EC – endemic control, AI – asymptomatic infected, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria.
Comparing group by group with the Endemic control: TNF was significantly lower in UM (p = 0,0006); IFN-γ was significantly higher in AI and CM (p = 0,02 and p = 0,007, respectively); IL-10 was significantly higher in UM (p = 0,0009), SM (p = 0,0001) and CM (p = 0,0001)

Table 4: Cytokine distribution in the Indian population: median TNF, IFN-γ and IL-10 per clinical group.

	NEC (min-max)	EC (min-max)	UM (min-max)	SM (min-max)	CM (min-max)	ExCM (min-max)
TNF (pg/ml)	57 (39–73)	78 (63–120)	180 (119–207)	200 (173–381)	530 (258–1227)	81 (69–124)
IFN-γ (pg/ml)	23 (10–31)	22 (17–201)	119 (70–153)	127 (111–200)	65 (45–101)	32 (11–53)
IL-10 (pg/ml)	13 (5–26)	22 (11–31)	120 (97–147)	176 (121–253)	301 (175–506)	85 (40–108)
IL-4 (pg/ml)	18 (8–40)	58 (23–84)	65 (31–204)	69 (50–89)	62 (40–85)	48 (29–80)

NEC – non endemic control, EC – endemic control, UM – uncomplicated malaria, SM – severe malaria (non-cerebral), CM – cerebral malaria, ExCM – Ex-cerebral malaria.
Comparing group by group with the Endemic control: TNF was significantly lower in NEC (p = 0,0006) and higher in UM (p = 1,19 × 10-7), SM (p = 1,01 × 10-5) and CM (p = 2,26 × 10-7); IFN-γ was significantly higher in all diseased groups (UM – p = 5,11 × 10-6, SM – p = 1,01 × 10-6, CM – p = 9,71 × 10-6); IL-10 was significantly higher in all diseased groups (UM – p = 1,04 × 10-7, SM – p = 1,01 × 10-5 and CM – p = 2,46 × 10-7).

levels are higher in UM and SM (Figure 1C and 1D) than in CM patients who had values similar to that of the controls [8,24]. In addition, the median levels of circulating IgE in the ExCM group were close to that of the CM group. Also, no significant correlation was found between both IgE and TNF levels in the CM group. These observations are contrary to published data describing increased IgE levels that correlate with high concentrations of circulating TNF, a cytokine associated with malaria severity and also with pRBC adherence on brain capillary endothelial cells [9,10,25,26]. The results suggest that either IgE does not play an important role in CM pathogenesis, or that these antibodies may participate in the parasite sequestration into the brain or other organ capillaries [24].

There was a significant increase in IgE levels with age in Gabonese children independent of the disease group (Figure 2). This increase in IgE production between 0 and six years of age may also reflect an increase in the capacity of the immune system to respond to parasite infections [7,27]. Such a correlation was not found in Indian groups. This would be expected because the Indian groups comprised mainly adults, with the few children being older than five years. Although most of individuals in the Gabonese endemic control group had already been in contact with the parasite, as demonstrated by the high titres of specific antibody to *P. falciparum*-infected red blood cells observed in these children, the median plasma IgE concentrations were compared with those of the Indian NEC group. Although the median total IgE levels in the Indian NEC groups was higher than that of the Gabonese EC group, suggesting that age could be an important factor in IgE production, the higher IgE levels in the Indian population may also be interpreted as the result of either environmental factors, such as predominance of food allergies, of the genetic background, which may predispose to developing IgE responses [13,27,29-32] or of a co-infection with other parasites, such as helminths affecting the IgE responses in these patients [33,34]. In the Gabonese cohorts, IgE levels tended to correlate negatively to parasitaemia except in UM whereas all patient groups from India showed a positive correlation.

The pool of circulating IgE comprises both monomeric and complexed immunoglobulins [35,36]. A functional test was performed, based on the ability of the circulating IgE from the sera of different patient groups to induce degranulation of mast cells in the presence of pRBC antigens to better estimate the *P. falciparum* specific IgE response. This test does not provide specific IgE concentration within total IgE. It is based on the specific IgE induced percentage of mast cells degranulation. Degranulation was measured by quantifying β-hexosaminidase release. Functional *P. falciparum*-specific IgEs were detected in randomly chosen patients from all groups in both the Gabonese and Indian populations, except for ExCM Indian patients. The highest percentage of patients with functional anti-parasite IgEs was found in the Gabonese AI and UM and Indian EC and UM groups, which decreased in the SM and CM groups (Figure 3A and 3B). This suggests a protective role for *P. falciparum*-specific IgE, and is consistent with previous published data [13,14].

Although the CM group had a low percentage of patients able to induce degranulation, it was the only group where there was one patient serum inducing a mast cell degranulation above 30%. This intense response may be associated with the presence of IgE with higher affinity for *P. falciparum* antigens, as previously reported by Gonzalez-Espinosa *et al.* [37]. However, the level of degranulation can also be enhanced by the number of receptors involved in recognizing the antigen-IgE complex, which can strongly affect the size of the secretory response [37,38]. There was no evident correlation between the level of functional *P. falciparum*-specific IgE (percentage of enzyme release) and the level of total IgE per group within each population. This is unsurprising, given that both the monomeric and complexed forms of circulating parasite-specific IgE can affect the level of degranulation [39-41]. It

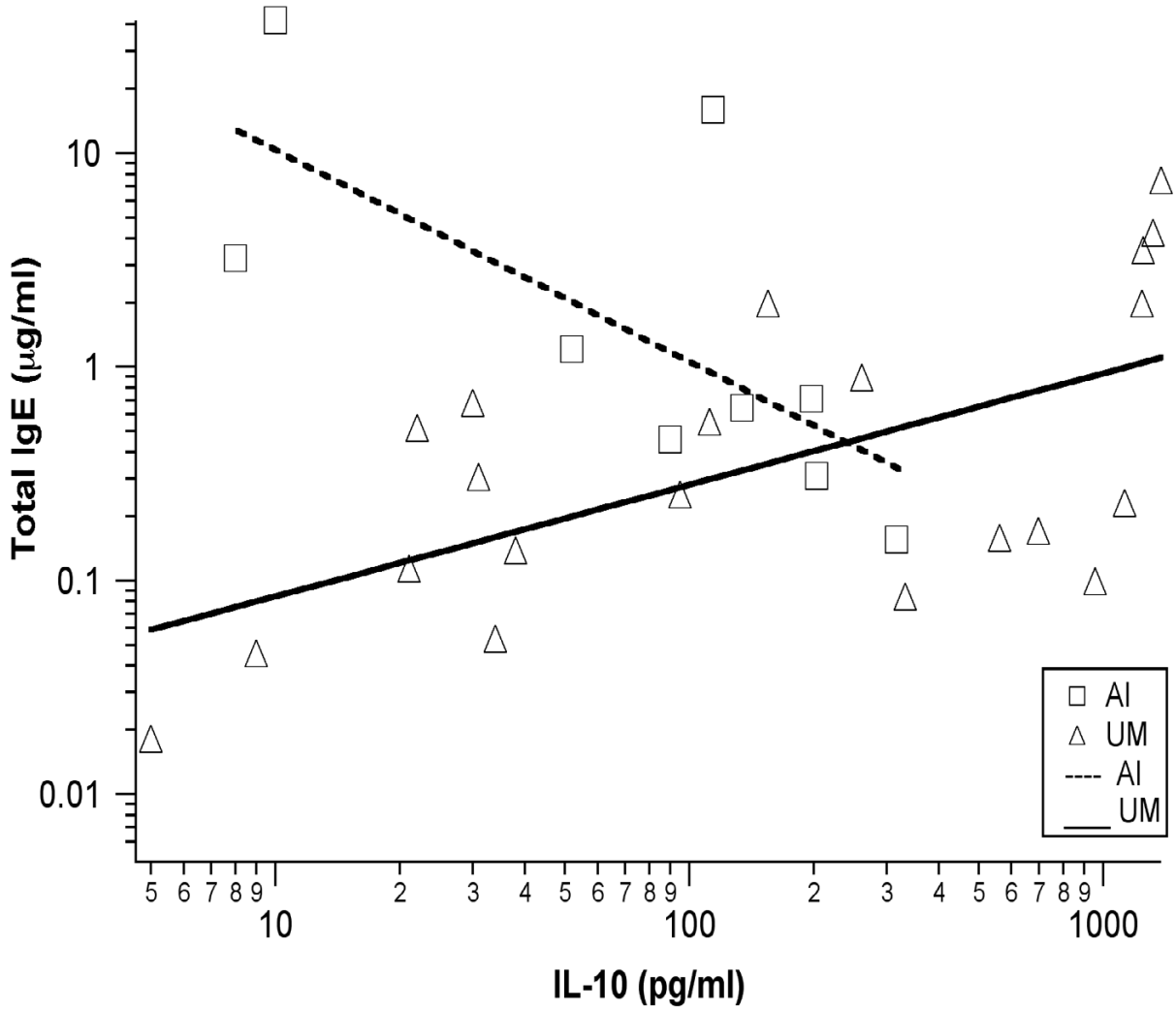


Figure 4
IL-10 correlation with total IgE levels in the Gabonese population. **Dashed line -** Asymptomatic patients (significant negative spearman correlation, p = 0.025). **Bold line -** Uncomplicated malaria patients (significant positive correlation, p = 0.017).

is also acceptable that total IgE levels were not directly correlated to FcåRI upregulation levels [40,42]. The minimum level of receptors occupied by the parasite antigen-specific IgE complex required to induce a degranulation response is 10%. Consequently, this response will be independent of the total IgE levels [20]. An increase in specific IgE levels has been also seen in other parasitic infections, such as helminthiasis, in which the specific IgEs usually help to eliminate the pathogens either through hypersensitivity reactions resulting from mast cell degranulation or by inducing antibody-dependent cell-mediated responses [27].

High levels of IgE in *P. falciparum*-infected individuals have been shown to be due to an underlying imbalance in favour of IL-4 in the ratio of $CD4^+$ T cell producers, which are responsible for the IgG/IgM isotype switching to IgE [10]. High levels of circulating IL-4 have also been associated with a greater parasite antigen-specific production of IgE in individuals less susceptible to malaria [13]. Also, Th1-type pro-inflammatory cytokines, such as IFN-γ and TNF are thought to play an important role in the both protecting against and increasing the pathogenesis of cerebral malaria [3,4,22]. Plasma TNF, IFN-γ and IL-10 levels were measured in both the Indian and Gabonese groups. In the

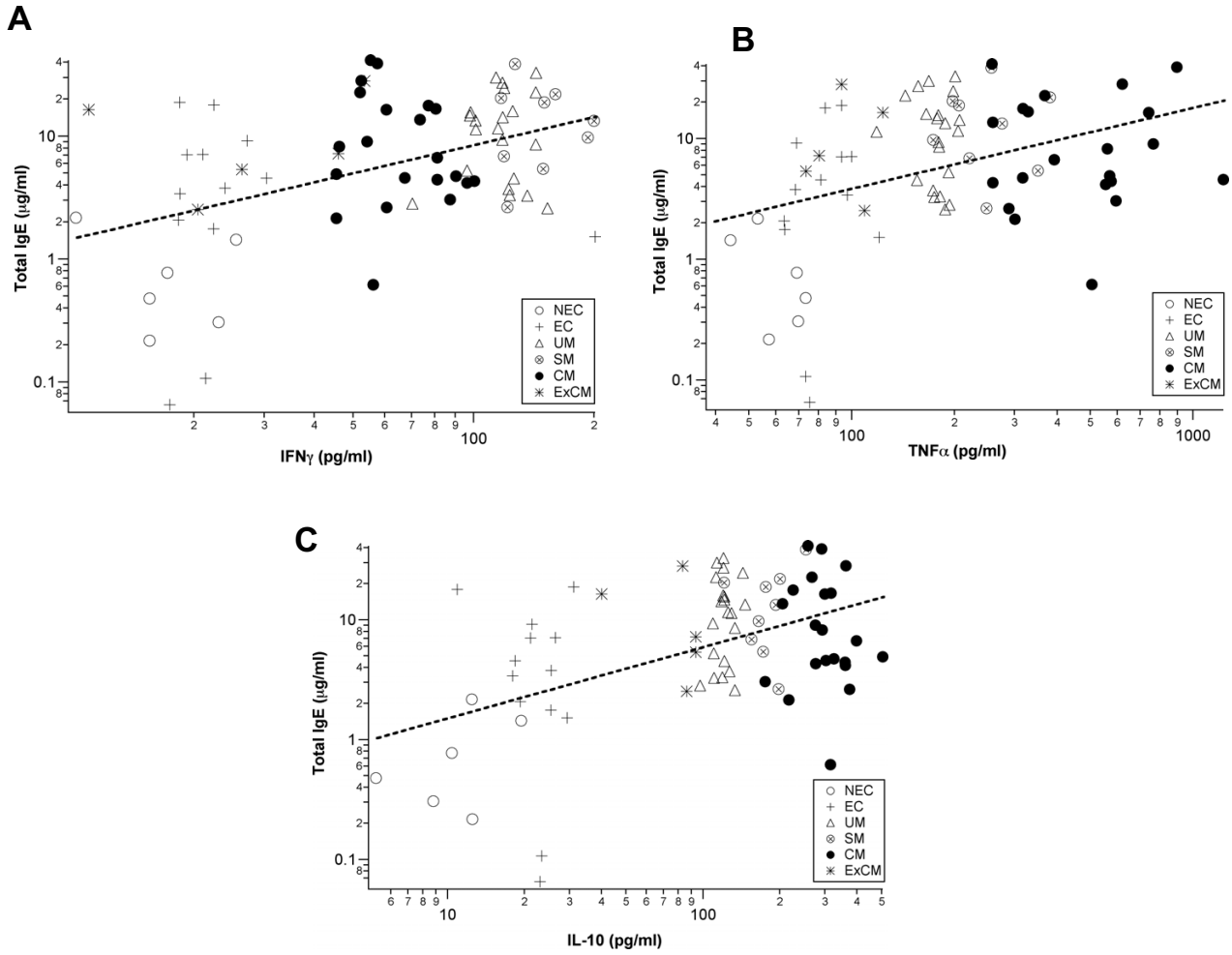


Figure 5
Cytokine correlation with IgE levels in the Indian population. **A.** TNF correlation with total IgE levels (significant positive spearman correlation, p = 0.0037); **B.** IFN-γ correlation with total IgE levels (Significant positive spearman correlation, p = 0.0028); **C**. IL-10 correlation with total IgE levels (significant positive spearman correlation, p = 0.0051).

Gabonese groups, no direct correlation was found between IgE and IFN-γ or TNF levels in the UM, SM and CM patients. However, the level of IgE production was correlated to IL-10 levels in UM and AI patients (Figure 4). In this group, there was a positive association between IL-10 levels and parasite load, but not between IgE levels and parasitaemia. In the Indian groups, IFN-γ, TNF and IL-10 levels were all significantly correlated with IgE levels independent of the group (Figures 5A, B and 5C). When looking for a correlation per group, no significant statistic value was found. IL-4 is the main cytokine responsible for IgE production, inducing antibody isotype switching from IgG and IgM to IgE [10]. Previous studies have shown an association between the IL-4/IFN-γ levels and IgE levels, suggesting an induced Th2-type switched response [43]. As IL-4 levels were different between groups only in Indians, a correlation was expected between this ratio and IgE levels, and surprisingly, the results showed an opposite correlation (p = 0,012). The levels of IL-4 remained almost constant across all groups as IgE levels increased. Therefore, this opposite correlation arises due to a parallel increase in both IFN-γ and IgE levels. This suggests that, in the Indian population, IL-4 does not seem to directly influence IgE levels. It has been shown experimentally that there are conditions under which alternative mechanisms may induce IgE production independent of IL-4 [44]. Regarding other cytokine ratios, like TNF/IL-10, no significant correlation was found with total IgE levels.

In conclusion, these results showed that total IgE levels increased in infected patients, mainly in UM and SM patients but not in CM patients. The decrease in total IgE levels in the CM group was associated with a higher intensity of mast cell degranulation induced *in vitro* by *P. falciparum*-specific IgE. In addition, no correlation was found between total and functional *P. falciparum*-specific IgE levels.

The results reported here show the high activity of functional circulating *P. falciparum*-specific IgE in asymptomatic malaria patients. However, no correlation was observed between plasma levels of total IgE, the disease phenotype and the cytokines pattern in the different groups of patients studied. The opposite association between pro- and anti-inflammatory cytokine ratios and IgE levels reveals the complexity of immune response disruption occurring during malaria in patients from low and high malaria endemic region.

Authors' contributions

Joana Duarte contributed to the acquisition, analysis, interpretation of data and manuscript drafting but not the design of the study. Prakash Deshpande and Vincent Guiyedi have highly contributed to conception and interpretation of data and manuscript revising. Salah Mécheri collaborated to the design and interpretation of IgE functional tests and manuscript drafting. Constantin Fesel contributed to the statistical analysis, interpretation of data and manuscript drafting.

Pierre-André Cazenave, Gyan C. Mishra and Maryvonne Kombila have contributed to conception of the study and manuscript revising. Sylviane Pied participated to conception, design, analysis and interpretation of data, the final approval of the version to be published.

Financial support

This work was part of the *Centre National de la Recherche Scientifique-Laboratoires Européens Associés* "*Génétique et developpement de la tolérance naturelle*" program. It was supported by the PAL+ program of the French Ministry of Research. We thank the Indo-French Centre for the Promotion of Advanced Research (IFCPAR), New Delhi, India for providing financial assistance (project No.2103-3). C-F received a post-doctoral fellowship from the *Fundaçâo para a Ciência e Tecnologia* (Portugal). V-G holds a fellowship from the *Agence Universitaire de la Francophonie* (AUF).

Acknowledgements

We thank M. Bouyou-Akotet and M. Idrissa-Boubou for assistance with the collection of plasma samples. We are grateful to R.O. for critical reading of and his help in preparing the manuscript.

References

1. Moorthy VS, Good MF, Hill AV: **Malaria vaccine developments.** *Lancet* 2004, **363:**150-156.
2. Miller LH, Baruch DI, Marsh K, Doumbo OK: **The pathogenic basis of malaria.** *Nature* 2002, **415:**673-679.
3. Heddini A: **Malaria pathogenesis: a jigsaw with an increasing number of pieces.** *Int J Parasitol* 2002, **32:**1587-1598.
4. de Souza JB, Riley EM: **Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis.** *Microbes Infect* 2002, **4:**291-300.
5. Adams S, Brown H, Turner G: **Breaking down the blood-brain barrier: signaling a path to cerebral malaria?** *Trends Parasitol* 2002, **18:**360-366.
6. Miller LH, Good MF, Milon G: **Malaria pathogenesis.** *Science* 1994, **264:**1878-1883.
7. Calissano C, Modiano D, Sirima BS, Konate A, Sanou I, Sawadogo A, Perlmann H, Troye-Blomberg M, Perlmann P: **IgE antibodies to *P. falciparum* and severity of malaria in children of one ethnic group living in Burkina Faso.** *Am J Trop Med Hyg* 2003, **69:**31-35.
8. Perlmann H, Helmby H, Hagstedt M, Carlson J, Larsson PH, Troye-Blomberg M, Perlmann P: **IgE elevation and IgE anti-malarial antibodies in *P. falciparum* malaria: association of high IgE levels with cerebral malaria.** *Clin Exp Immunol* 1994, **97:**284-292.
9. Perlmann P, Perlmann H, ElGhazali G, Blomberg MT: **IgE and tumor necrosis factor in malaria infection.** *Immunol Lett* 1999, **65:**29-33.
10. Perlmann P, Perlmann H, Flyg BW, Hagstedt M, Elghazali G, Worku S, Fernandez V, Rutta AS, Troye-Blomberg M: **Immunoglobulin E, a pathogenic factor in *P. falciparum* malaria.** *Infect Immun* 1997, **65:**116-121.
11. Perlmann P, Perlmann H, Looareesuwan S, Krudsood S, Kano S, Matsumoto Y, Brittenham G, Troye-Blomberg M, Aikawa M: **Contrasting functions of IgG and IgE antimalarial antibodies in uncomplicated and severe *P. falciparum* malaria.** *Am J Trop Med Hyg* 2000, **62:**373-377.
12. Seka-Seka J, Brouh Y, Yapo-Crezoit AC, Atseye NH: **The role of serum immunoglobulin E in the pathogenesis of *P. falciparum* malaria in Ivorian children.** *Scand J Immunol* 2004, **59:**228-230.
13. Bereczky S, Montgomery SM, Troye-Blomberg M, Rooth I, Shaw MA, Farnert A: **Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria.** *Int J Parasitol* 2004, **34:**935-942.
14. Farouk SE, Dolo A, Bereczky S, Kouriba B, Maiga B, Farnert A, Perlmann H, Hayano M, Montgomery SM, Doumbo OK, Troye-Blomberg M: **Different antibody- and cytokine-mediated responses to *P. falciparum* parasite in two sympatric ethnic tribes living in Mali.** *Microbes Infect* 2005, **7:**110-117.
15. Oettgen HC, Geha RS: **IgE regulation and roles in asthma pathogenesis.** *J Allergy Clin Immunol* 2001, **107:**429-440.
16. Nyakeriga MA, Troye-Blomberg M, Bereczky S, Perlmann H, Perlmann P, ElGhazali G: **Immunoglobulin E (IgE) containing complexes induce IL-4 production in human basophils: effect on Th1-Th2 balance in malaria.** *Acta Trop* 2003, **86:**55-62.
17. Reischl IG, Coward WR, Church MK: **Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE-FcepsilonRI) interaction.** *Biochem Pharmacol* 1999, **58:**1841-1850.
18. WHO: **Severe and complicated malaria.** *Trans R Soc Trop Med Hyg* 1990, **84:**1-65.
19. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193:**673-675.
20. Marchand F, Mecheri S, Guilloux L, Iannascoli B, Weyer A, Blank U: **Human serum IgE-mediated mast cell degranulation shows poor correlation to allergen-specific IgE content.** *Allergy* 2003, **58:**1037-1043.
21. Takagi K, Nakamura R, Teshima R, Sawada J: **Application of human Fc epsilon RI alpha-chain-transfected RBL-2H3 cells for estimation of active serum IgE.** *Biol Pharm Bull* 2003, **26:**252-255.
22. Plebanski M, Hill AV: **The immunology of malaria infection.** *Curr Opin Immunol* 2000, **12:**437-441.
23. Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G: **Role of Th1 and Th2 cytokines in immune response to uncomplicated *P. falciparum* malaria.** *Clin Diagn Lab Immunol* 2002, **9:**348-351.
24. Maeno Y, Perlmann P, Perlmann H, Kusuhara Y, Taniguchi K, Nakabayashi T, Win K, Looareesuwan S, Aikawa M: **IgE deposition in**

brain microvessels and on parasitized erythrocytes from cerebral malaria patients. *Am J Trop Med Hyg* 2000, **63:**128-132.
25. Artavanis-Tsakonas K, Tongren JE, Riley EM: **The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology.** *Clin Exp Immunol* 2003, **133:**145-152.
26. Chen Q, Schlichtherle M, Wahlgren M: **Molecular aspects of severe malaria.** *Clin Microbiol Rev* 2000, **13:**439-450.
27. Winter WE, Hardt NS, Fuhrman S: **Immunoglobulin E: importance in parasitic infections and hypersensitivity responses.** *Arch Pathol Lab Med* 2000, **124:**1382-1385.
28. Migot-Nabias F, Mombo LE, Luty AJ, Dubois B, Nabias R, Bisseye C, Millet P, Lu CY, Deloron P: **Human genetic factors related to susceptibility to mild malaria in Gabon.** *Genes Immun* 2000, **1:**435-441.
29. Poulsen LK: **Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods.** *Mol Nutr Food Res* 2004, **48:**413-423.
30. Yazdanbakhsh M, Kremsner PG, van Ree R: **Allergy, parasites, and the hygiene hypothesis.** *Science* 2002, **296:**490-494.
31. Paganotti GM, Babiker HA, Modiano D, Sirima BS, Verra F, Konate A, Ouedraogo AL, Diarra A, Mackinnon MJ, Coluzzi M, Walliker D: **Genetic complexity of *P. falciparum* in two ethnic groups of Burkina Faso with marked differences in susceptibility to malaria.** *Am J Trop Med Hyg* 2004, **71:**173-178.
32. Verra F, Luoni G, Calissano C, Troye-Blomberg M, Perlmann P, Perlmann H, Arca B, Sirima BS, Konate A, Coluzzi M, Kwiatkowski D, Modiano D: **IL4-589C/T polymorphism and IgE levels in severe malaria.** *Acta Trop* 2004, **90:**205-209.
33. Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, Vouldoukis I, Looareesuwan S: ***Ascaris lumbricoides* infection is associated with protection from cerebral malaria.** *Parasite Immunol* 2000, **22:**107-113.
34. Nacher M, Singhasivanon P, Traore B, Vannaphan S, Gay F, Chindanond D, Franetich JF, Mazier D, Looareesuwan S: **Helminth infections are associated with protection from cerebral malaria and increased nitrogen derivatives concentrations in Thailand.** *Am J Trop Med Hyg* 2002, **66:**304-309.
35. Johansson SG: **Anti-IgE antibodies in human serum.** *J Allergy Clin Immunol* 1986, **77:**555-557.
36. Marone G, Spadaro G, Palumbo C, Condorelli G: **The anti-IgE/anti-FcepsilonRIalpha autoantibody network in allergic and autoimmune diseases.** *Clin Exp Allergy* 1999, **29:**17-27.
37. Gonzalez-Espinosa C, Odom S, Olivera A, Hobson JP, Martinez ME, Oliveira-Dos-Santos A, Barra L, Spiegel S, Penninger JM, Rivera J: **Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells.** *J Exp Med* 2003, **197:**1453-1465.
38. Posner RG, Paar JM, Licht A, Pecht I, Conrad DH, Hlavacek WS: **Interaction of a monoclonal IgE-specific antibody with cell-surface IgE-Fc epsilon RI: characterization of equilibrium binding and secretory response.** *Biochemistry* 2004, **43:**11352-11360.
39. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G: **Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival.** *Immunity* 2001, **14:**801-811.
40. Kitaura J, Xiao W, Maeda-Yamamoto M, Kawakami Y, Lowell CA, Kawakami T: **Early divergence of Fc epsilon receptor I signals for receptor up-regulation and internalization from degranulation, cytokine production, and survival.** *J Immunol* 2004, **173:**4317-4323.
41. Yamaguchi M, Sayama K, Yano K, Lantz CS, Noben-Trauth N, Ra C, Costa JJ, Galli SJ: **IgE enhances Fc epsilon receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc epsilon receptor I expression and mediator release.** *J Immunol* 1999, **162:**5455-5465.
42. Saini SS, Klion AD, Holland SM, Hamilton RG, Bochner BS, Macglashan DW: **The relationship between serum IgE and surface levels of FcepsilonR on human leukocytes in various diseases: correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils.** *J Allergy Clin Immunol* 2000, **106:**514-520.
43. Elghazali G, Perlmann H, Rutta AS, Perlmann P, Troye-Blomberg M: **Elevated plasma levels of IgE in *P. falciparum* -primed individuals reflect an increased ratio of IL-4 to interferon-gamma (IFN-gamma)-producing cells.** *Clin Exp Immunol* 1997, **109:**84-89.
44. Grunewald SM, Teufel M, Erb K, Nelde A, Mohrs M, Brombacher F, Brocker EB, Sebald W, Duschl A: **Upon prolonged allergen exposure IL-4 and IL-4Ralpha knockout mice produce specific IgE leading to anaphylaxis.** *Int Arch Allergy* 2001, **125:**322-328.

Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide

Meri K. Tulić, Janet L. Wale, Patrick G. Holt, and Peter D. Sly

TVW Telethon Institute for Child Health Research; and University of Western Australia Department of Paediatrics, Perth, Australia

The potential role of respiratory infections in altering the development of atopy and asthma is complex. Infections have been suggested to be effective in preventing the induction of T-helper 2–polarized allergen-specific immunity in early life, but also to exacerbate asthma in older, sensitized individuals. The mechanism(s) underlying these effects are poorly defined. The aim of this work was to determine the influence of lipopolysaccharide (LPS) exposure on the development of sensitization to allergen and the response to allergen challenge *in vivo.* Piebald–Virol–Glaxo rats were exposed to a single aerosol of LPS 1 d before or 1, 2, 4, 6, 8, or 10 d after sensitization with ovalbumin (OVA). On Day 11 animals were exposed to 1% OVA and responses to allergen were measured 24 h later, monitoring inflammatory cell influx and microvascular leakage into bronchoalveolar lavage (BAL) fluid as well as pulmonary responses to methacholine using the forced oscillation technique. Histologic analysis was included to complement the BAL results. Single aerosol exposure to LPS 1 d before and up to 4 d after intraperitoneal injection of OVA protected against the development of OVA-specific immunoglobulin (Ig) E. LPS exposure 6, 8, or 10 d after sensitization further exacerbated the OVA-induced cellular influx, resulting in neutrophilia and increased Evans Blue dye leakage with no effect on serum IgE levels. In addition, LPS abolished the OVA-induced hyperresponsiveness in sensitized animals when given 18 h after OVA challenge. This study demonstrates that exposure to LPS can modify the development of allergic inflammation *in vivo* by two independent mechanisms. Exposure early in the sensitization process, up to Day 6 after exposure to allergen, prevented allergen sensitization. Exposure to LPS after allergen challenge in sensitized animals abolished the hyperresponsiveness and modified the inflammatory cell influx characteristic of late-phase response to allergen.

Asthma is recognized as a chronic inflammatory disorder of the lungs that results in bronchial hyperresponsiveness, deterioration of lung function, and impaired quality of life if left untreated (1, 2). Allergic sensitization and respiratory symptoms on exposure to allergen form an integral part of the clinical spectrum of asthma in many older children and adults. The histologic appearances of the airways are similar in both atopic and intrinsic asthmatics (3) and serum immunoglobulin (Ig) E levels are raised (4). Although atopy is not always linked with asthma, its importance increases with age (5). Microbial infections early in infancy may protect from the later development of atopy and asthma such that the stimulus for normal postnatal maturation of the immunoinflammatory response may be provided by microbial stimulation (6, 7). At the same time, clinical data indicate that respiratory viral infections are major trigger factors for acute exacerbations of asthma (8–10). There is circumstantial evidence that some bacterial infections, for example *mycoplasma* and *chlamydia* may also play a role in precipitating asthma (11, 12); however, the clinical impression is that bacterial pneumonia in asthmatic patients does not trigger acute exacerbations.

The inflammation characteristics of asthma and respiratory infections are different. Two subsets of T-helper (Th) lymphocytes have been defined and are distinguishable by a differing pattern of cytokine release. Th1-type cells stimulate the recruitment and activity of macrophages and/or mononuclear phagocytes and are involved in the cellular immunity activated by microbial antigens, typically resulting in IgG antibody production without an increase in IgE (13). They produce interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factors. Th2 responses are important in the allergic response and asthma, producing the cytokines IL-4, -5, -6, -10, and -13 that are involved in the humoral immune responses of elevated IgE levels and eosinophilia (13). Complex, antagonistic interrelationships exist between the two subsets and the cytokine milieu (13–15).

On the basis of epidemiologic data suggesting that nonwheezing infections may protect against allergen sensitization and also of clinical impressions that bacterial infections do not trigger asthma, we hypothesized that exposure to the bacterial product lipopolysaccharide (LPS) during the usual time course of late-phase allergic reactions would alter the late-phase events. To address this question we used two *in vivo* models of inflammatory lung disease measuring inflammatory cell influx into bronchoalveolar lavage (BAL) fluid (BALF) and leakage of Evans Blue dye from the microvasculature into the BALF, and monitoring the responsiveness to methacholine in Piebald–Virol–Glaxo (PVG) rats sensitized to ovalbumin (OVA). PVG rats also demonstrate neutrophilia and increased vascular permeability after a single aerosol exposure to LPS.

Materials and Methods

Animals

Male PVG rats weighing 200 to 250 grams (Research Centre, Institute for Child Health Research, Perth, Australia) were used and were 10 wk old at the time of data collection. They were barrier-housed in a clean animal house environment, kept on an OVA-free diet, and had access to water and food *ad libitum*. An-

(*Received in original form February 24, 1999 and in revised form December 1, 1999*)

Address correspondence to: Ms. Meri Katarina Tulić, Div. of Clinical Sciences, TVW Telethon Institute for Child Health Research, P.O. Box 855, West Perth, WA 6872, Australia. E-mail: merit@ichr.uwa.edu.au

Abbreviations: bronchoalveolar lavage, BAL; BAL fluid, BALF; interferon, IFN; immunoglobulin, Ig; interleukin, IL; lipopolysaccharide, LPS; methacholine, MCh; ovalbumin, OVA; phosphate-buffered saline, PBS; provocative concentration of MCh producing a 150% increase in frequency-dependent tissue resistance above baseline, PC_{150}; Piebald–Virol–Glaxo rats, PVG rats; standard error of the mean, SEM; T-helper, Th; input impedance, Z_L.

Am. J. Respir. Cell Mol. Biol. Vol. 22, pp. 604–612, 2000
Internet address: www.atsjournals.org

nual monitoring indicated that the colony was free of known pathogens. The study protocol was approved by the Institutional Animal Ethics Committee.

Sensitization Procedure

Eight-week-old rats were actively sensitized on Day 0 by a single intraperitoneal injection with 100 μg OVA in phosphate-buffered saline (PBS), along with 50 ng of the IgE-selective adjuvant ricin.

Exposure to LPS

Where appropriate, animals were exposed once to nebulized LPS *Salmonella typhimurium* (50 $\mu g \cdot ml^{-1}$) for 30 min by allowing them to run freely in a Plexiglas chamber into which the LPS was aerosolized. The aerosol was generated from an ultrasonic nebulizer (De Vilbiss Ultra-Neb 2000; Sunrise Medical, Somerset, PA), the outlet of which was connected to the chamber. The output of the nebulizer was 0.5 $ml \cdot min^{-1}$ and the mean particle size was 3.5 μm (manufacturer's specifications). Naive animals were exposed to LPS either 6 or 24 h before measurements were taken, whereas sensitized animals underwent exposure 1 d before or 1, 2, 4, 6, 8, 10, or 12 d after sensitization with allergen. A second group of sensitized animals were exposed to LPS 18 h after OVA challenge.

Allergen Challenge

At 11 d after sensitization, at the peak of their IgE response, the animals were placed in a Plexiglas chamber and challenged with aerosolized OVA (1%) for 30 min using the ultrasonic nebulizer (De Vilbiss).

Animal Preparation

At 24 h after allergen challenge, the animals were anesthetized by intramuscular injection of xylazine (12 $mg \cdot kg^{-1}$) and ketamine (40 $mg \cdot kg^{-1}$) and a tracheostomy was performed. The femoral vein was cannulated with polyethylene tubing for the intravenous injection of drugs. Evans Blue dye (50 $mg \cdot kg^{-1}$) in a volume of 2 ml was administered by intravenous injection over a 2-min period.

Measurement of Respiratory Mechanics

Pulmonary function was measured using an adaptation of the low-frequency forced oscillation technique, in which input impedance (Z_L) was measured using a wave-tube (16, 17). Briefly, measurements were made by applying loudspeaker-generated small-amplitude oscillatory signals from 0.5 to 20 Hz through a 114-cm-long, 1.45-mm inner diameter polyethylene wave tube during a 6-s apnoeic period. A three-way tap was used to switch the animal from the respirator to a loudspeaker-in-box system at end-expiration. The mean pressure in the loud speaker was adjusted to 2.5 cm H_2O to keep the transpulmonary pressure constant during measurements. Z_L was calculated as described by Peták and colleagues (16). To separate airway and tissue mechanics, a model containing a frequency-independent airway resistance and inertance and a constant-phase frequency-dependent tissue resistance (G) and elastance was fitted to the Z_L spectra (17). Impedance points coinciding with the heart rate and its harmonics were omitted from the model fitting because cardiac activity caused low signal-to-noise ratio at these frequency components.

Methacholine Challenge

After an equilibration period of 15 min, baseline was established with four to six Z_L recordings. Cumulative doses of methacholine (MCh) (2 to 16 $mg \cdot ml^{-1}$) were administered by inhalation for 90 s using a jet nebulizer (LC PLUS; Pari-Werk GmbH, Starnberg, Germany) driven by compressed air (5 $liters \cdot min^{-1}$) and connected to the input port of the ventilator. Z_L data were ensemble-averaged in baseline condition, whereas individual Z_L curves were fitted at 1-min intervals after MCh administration. Peak responses in G at each dose were used for further analysis. Responses were measured as percentage increase above baseline.

Evans Blue dye (50 $mg \cdot kg^{-1}$) was administered by intravenous injection over a 2-min period immediately after the construction of MCh dose–response curves. At the end of the experiment, 1.0 ml of blood was collected via cardiac puncture for the estimation of OVA-specific serum antibody titers.

BAL

At 30 min after administration of Evans Blue, animals were killed with pentobarbitone and the chest was opened. BAL was performed via the tracheal cannula using 3 × 8 ml of PBS containing lignocaine hydrochloride (0.35%) and bovine serum albumin (BSA) (0.2%). Routinely, greater than 90% of the lavaging fluid was recovered from the lungs. The BALF was centrifuged at 250 × *g* for 10 min at 4°C and the cell pellet resuspended in 1.0 ml sterile PBS solution. Total cell count was determined by adding 20 μl of the cell suspension to 20 μl Trypan Blue stain and counting cells under a light microscope using a Neubauer hemocytometer. The differential cell count was carried out from cytospin preparations using Leishman's BDH stain and counting 200 cells at random under ×100 magnification. The cells were identified by standard morphology.

The amount of Evans Blue dye in the lavage supernatant was quantified by measuring the absorbence at 630 nm using a spectrophotometer (Microplate AutoReader EL311; Bio-Tek Instruments, Inc., Winooski, VT). The concentration of dye was extrapolated from a standard curve of Evans Blue dye concentrations (1 to 10 $\mu g \cdot ml^{-1}$).

Serum Antibody Measurements

OVA-specific IgE titers were estimated by enzyme-linked immunosorbent assay (ELISA) according to the method of Van Halteren and associates (18). Briefly, microtiter plates (Falcon flexible assay plate 3912; Becton Dickinson, Winooski, CA) were coated overnight at 4°C with mouse antirat IgE in PBS (1 $\mu g \cdot ml^{-1}$). Doubling dilutions of standards and samples were added and incubated for 3 h. Digoxygenin conjugate OVA was added (1:1,000) and incubated for 1 h, followed by sheep antidigoxygenin horseradish peroxidase conjugate (1:1,000) for 1 h. The peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was used for color development and the plates were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate AutoReader EL311; Bio-Tek Instruments). Titers are expressed as reciprocal $\log_2$ titers. OVA-specific IgG was measured by a hemagglutination assay using sheep red blood cells conjugated to OVA and titers expressed as reciprocal $\log_2$ titers as previously described (19).

Histologic Analysis

In a separate group of animals histologic examination of the lungs was undertaken to determine whether the inflammatory cell profile obtained using the BALF was representative of tissue inflammation. Sensitization and OVA challenge as well as LPS exposure were performed as described earlier. At 6 h after LPS exposure in naive animals or on Day 12, 24 h after OVA challenge in sensitized animals, the rats were killed and the lungs and the trachea removed immediately and inflation-fixed with 4% paraformaldehyde at 20 cm H_2O via tracheal installation. The right lung and the trachea were embedded in paraffin wax and one 3-μm section per animal was stained with hematoxylin and eosin. Inflammatory cells were identified by standard morphometry. Numbers of cells in tissue and air spaces were counted in 10 random, nonoverlapping parenchymal fields at ×100 magnification under brightfield

TABLE 1

*Effects of OVA on inflammatory cell count, microvascular leakage, and serum antibody levels**

Treatment	*n*	OVA-Specific IgE ($1/log_2$ *titer*)	OVA-Specific IgG ($1/log_2$ *titer*)	Total Cells ($10^6/ml$)	Eosinophils ($10^6/ml$)	Macrophages ($10^6/ml$)	Lymphocytes ($10^6/ml$)	Neutrophils ($10^6/ml$)	Evans Blue (*mg/ml*)
Naive	11	3.50 ± 0.24	3.59 ± 0.43	0.87 ± 0.08	0	0.72 ± 0.07	0.12 ± 0.02	0.03 ± 0.01	1.98 ± 0.10
Sens/sal	8	7.32 ± 0.29§	4.32 ± 0.43	0.64 ± 0.14	0	0.44 ± 0.02	0.18 ± 0.04	0.02 ± 0.01	1.95 ± 0.02
Sens/OVA	11	7.03 ± 0.35	4.15 ± 0.40	4.16 ± 0.19†	0.16 ± 0.03†	1.59 ± 0.10†	2.15 ± 0.15†	0.26 ± 0.03†	4.36 ± 0.21†

*Inflammatory response to OVA in sensitized animals 24 h after allergen exposure (sens/OVA group). Serum OVA–specific IgE and IgG were measured. The total and the differential inflammatory cell count as well as microvascular leakage (assessed by Evans Blue dye) was studied using the BALF. Results are expressed as means ± SEM.

§$P < 0.01$ versus naive.

†$P < 0.01$ versus sensitized and saline-challenged animals (sens/sal group).

illumination. All histologic analyses was performed on the same region of each lung, the right upper lobe, and measurements were performed blind by the same operator (M.K.T.).

Study Groups

Influence of LPS exposure on late-phase events after OVA challenge. Six sensitized animals were exposed to a single aerosol challenge of saline or LPS 18 h after OVA challenge on Day 12 and inflammatory parameters as well as responses to inhaled MCh were determined 6 h later; that is, 24 h after initial allergen exposure.

Influence of LPS exposure on primary allergic sensitization. Groups of animals were exposed to saline or LPS 1 d before or 1, 2, 4, 6, 8, or 10 d after sensitization with intraperitoneal injection of OVA. They were then challenged with 1% OVA on Day 11 and their serum and BALF collected 24 h later.

Histologic analysis. Six key groups of rats (4 in each group) were exposed to saline or LPS in naive or sensitized animals to complement the BAL data with parenchymal sections. These animals were not lavaged.

Drugs and Materials

OVA (Grade V), ricin, LPS *S. typhimurium*, acetyl-β-MCh chloride (MCh), lignocaine hydrochloride, Evans Blue dye, Trypan Blue, and Leishman's BDH stain were obtained from Sigma Chemical Company (St. Louis, MO). Mouse (monoclonal) antirat IgE was supplied by Biosource (Camarillo, CA); digoxygenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester, sheep antidigoxygenin-POD Fab fragments by Roche Diagnostics (Basel, Switzerland); and TMB peroxidase substrate and Solution B by Kirkegaard & Perry Laboratories (Gaithersburg, MD). BSA was from CSL (Parkville, Australia) and RPMI 1640 from GIBCO BRL (Glen Waverley, Australia). Ketamine (Ketamil) was purchased from Troy Laboratories (Smithfield, Australia), xylazine (Rompun) from Bayder (Pymble, Australia), and pentobarbitone sodium (Lethabarb) from Virbac (Peakhurst, Australia). Paraformaldehyde and Depex mounting medium were purchased from BDH Laboratory Supplies (Poole, UK).

Statistical Analysis

Measurement of the difference in the total and the differential cell counts, as well as Evans Blue leakage and serum antibody measurements between different treatment groups were made by the simple unpaired two-tailed Student's *t* test. The effects of LPS treatment in naive and sensitized animals on MCh responses were assessed by calculating the provocative concentration of MCh producing a 150% increase in frequency-dependent tissue resistance above baseline (PC_{150}) by linear interpolation on a semilogarithmic MCh dose–response curve. In this particular animal model aerosolized MCh produces predominantly a tissue response (16), thus only tissue mechanics were used for statistical analysis. Comparisons between groups were made on log transformed data using one-way analysis of variance with Student–Newman–Keuls correction for multiple comparisons. All results are expressed as means ± standard error of the mean (SEM). $P < 0.05$ was regarded as statistically significant.

Results

Inflammatory Models

Response to allergen in sensitized animals 24 h after challenge. Intraperitoneal sensitization of naive animals to OVA resulted in increased OVA-specific serum IgE on Day 12 ($P < 0.01$, $n = 8$; Table 1). OVA-specific serum IgG antibody was unchanged. Exposure of these animals to 1% OVA on Day 11 resulted in a greater than 4-fold increase in inflammatory cell influx into the BALF 24 h later ($P < 0.01$, $n = 11$) as a result of increased numbers of eosinophils ($P < 0.01$), macrophages ($P < 0.01$), lymphocytes ($P < 0.01$), and neutrophils ($P < 0.01$). This cellular influx was associated with a significant increase in the concentration of Evans Blue in BALF ($P < 0.01$; Table 1).

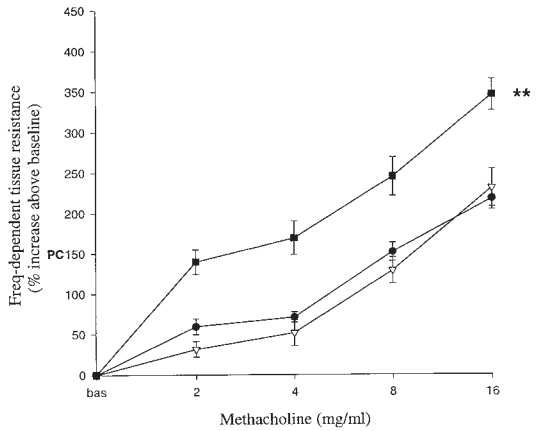


Figure 1. Effects of OVA in sensitized animals. Cumulative dose–response curves to inhaled MCh (2 to 16 mg · ml^{-1}) in naive (*filled circles*) ($n = 9$) or in sensitized and OVA-challenged animals (sens/OVA) (*filled squares*) ($n = 11$). Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. **$P < 0.01$ versus sensitized and saline-challenged animals (sens/sal) (*open inverted triangles*) ($n = 10$).

TABLE 2

*Effects of LPS on inflammatory cell count, microvascular leakage, and serum antibody levels**

Treatment	*n*	Total Cells (10^6/ml)	Neutrophils (10^6/ml)	Macrophages (10^6/ml)	Lymphocytes (10^6/ml)	Eosinophils (10^6/ml)	OVA-Specific IgE ($1/\log_2$ titer)	OVA-Specific IgG ($1/\log_2$ titer)	Evans Blue (mg/ml)
Naive	11	0.87 ± 0.08	0.03 ± 0.01	0.72 ± 0.07	0.12 ± 0.02	0	3.50 ± 0.24	3.59 ± 0.43	1.98 ± 0.10
LPS6	10	4.72 ± 0.34†	4.14 ± 0.30†	0.39 ± 0.05†	0.19 ± 0.05	0	3.42 ± 0.25	3.92 ± 0.53	3.47 ± 0.44†
LPS24	9	2.39 ± 0.31†	2.04 ± 0.26†	0.25 ± 0.04†	0.09 ± 0.01	0	3.10 ± 0.29	9.10 ± 0.43†	2.52 ± 0.36

Definition of abbreviations: LPS6 = LPS exposure on Day 6; LPS24 = LPS exposure on Day 24.

*Inflammatory response to 50 mg/ml LPS 6 or 24 h after challenge in naive animals. Total and differential inflammatory cell counts and microvascular leakage (assessed by Evans Blue dye) were studied using the BALF. IgE and IgG levels were monitored from serum. Results are expressed as means ± SEM.

†$P < 0.01$ versus naive.

OVA exposure induced hyperresponsiveness to inhaled MCh in sensitized animals, significantly shifting the dose–response curve to the left and decreasing the PC_{150} from 10.12 ± 1.16 to 3.39 ± 0.68 mg · ml^{-1} ($P < 0.01$; Figure 1).

Response to LPS in naive animals. **Six hours after exposure**. Exposure of naive animals to LPS (50 μg · ml^{-1}) induced a greater than 5-fold increase in the total number of inflammatory cells present in the BALF 6 h after initial exposure ($P < 0.01$, $n = 10$), predominantly as a result of neutrophil influx ($P < 0.01$; Table 2), making up 88% of the total cell count. The number of macrophages was reduced 2-fold ($P < 0.01$); lymphocyte number and serum OVA–specific IgE/IgG antibody levels remained unchanged (Table 2). Evans Blue leakage increased significantly from 1.98 ± 0.14 to 3.47 ± 0.44 mg · ml^{-1} ($P < 0.01$; Table 2). Responses to inhaled MCh were significantly potentiated 6 h after LPS exposure, decreasing the PC_{150} from 8.75 ± 1.10 to 2.66 ± 0.37 mg · ml^{-1} ($P < 0.01$; Figure 2). **Twenty-four hours after exposure**. At 24 h after initial exposure to LPS (50 μg · ml^{-1}), the total number of cells in BALF remained significantly above the pre-exposure number ($P < 0.01$, $n = 9$); however, this was reduced 2-fold when compared with 6-h exposure (Table 2). A similar pattern of cellular response was observed, neutrophils making up 85% of the cellular population. Macrophage numbers were reduced ($P < 0.01$) and lymphocyte numbers remained unchanged (Table 2). OVA-specific IgG was significantly increased from 3.92 ± 0.53 to 9.10 ± 0.43 $1/\log_2$ titer 24 h after LPS exposure ($P < 0.01$; Table 2). The increased Evans Blue leakage (Table 2) and the hyperresponsiveness evident at 6 h after LPS exposure was no longer evident at 24 h after exposure with a PC_{150} of 11.34 ± 1.81 mg · ml^{-1} (Figure 2).

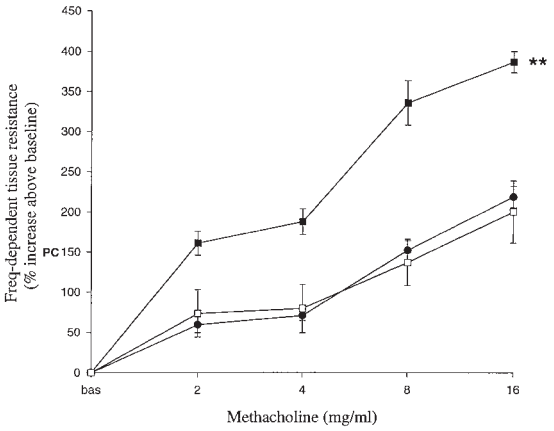


Figure 2. Effects of LPS in naive animals. Cumulative dose–response curves to inhaled MCh (2 to 16 mg · ml^{-1}) in naive animals (*filled circles*) ($n = 9$) exposed to 50 μg · kg^{-1} LPS 6 (*filled squares*) ($n = 10$) or 24 (*open squares*) ($n = 9$) h before data collection. Results are measured as a percentage increase in frequency-dependent tissue resistance and expressed as means ± SEM. **$P < 0.01$ versus naive animals.

Influence of LPS Exposure on Allergen-Induced Late-Phase Events

Exposure of sensitized animals to LPS on Day 12, 18 h after allergen challenge (denoted as the sens/OVA/LPS group), resulted in modification of the allergen-induced inflammatory cell profile seen in the BALF 24 h after allergen challenge. LPS further potentiated the allergen-induced inflammatory cell influx into BALF ($P < 0.05$, $n = 6$; Table 3). However, in contrast to the allergen challenge in sensitized animals in the absence of LPS (the sens/OVA/sal group), this increase in total cell number was predominantly due to a 20-fold increase in neutrophil influx ($P < 0.01$) making up greater than 80% of the cellular content. The numbers of both macrophages ($P < 0.01$) and lymphocytes ($P < 0.01$) were reduced 3-fold whereas eosinophils were no longer detected in the BALF ($P < 0.01$; Table 3). The OVA-specific serum antibody counts remained unchanged (Table 3). The allergen-induced leakage of Evans Blue into BALF in sensitized animals was further exacerbated with LPS exposure ($P < 0.01$; Table 3). LPS attenuated the allergen-induced hyperresponsiveness to MCh in sensitized animals when given 18 h after OVA challenge (Figure 3), shifting the dose–response curve to the right and increasing the PC_{150} from 2.62 ± 0.61 mg · ml^{-1} in the sens/OVA/sal-challenged control group to 10.77 ± 3.58 mg · ml^{-1} in the sens/OVA/LPS group ($P < 0.05$; Figure 3). LPS exposure in the sensitized and saline-challenged (as opposed to OVA) group (denoted as sens/sal/LPS in Figure 3) showed hyperresponsiveness to MCh (PC_{150} 2.09 ± 0.20 mg · ml^{-1}), and these responses were similar in magnitude to those in naive animals exposed to LPS 6 h before measurement (Figure 2).

TABLE 3

*Effects of LPS in naive animals on inflammatory cell count, microvascular leakage, and serum antibody levels**

Treatment	*n*	Total Cells (10^6/ml)	Neutrophils (10^6/ml)	Macrophages (10^6/ml)	Lymphocytes (10^6/ml)	Eosinophils (10^6/ml)	OVA-Specific IgE ($1/log_2$ titer)	OVA-Specific IgG ($1/log_2$ titer)	Evans Blue (mg/ml)
Sens/sal/LPS	6	4.90 ± 0.39	4.32 ± 0.37	0.34 ± 0.04	0.24 ± 0.03	0	6.99 ± 0.37	4.15 ± 0.34	3.65 ± 0.42
Sens/OVA/sal	6	4.53 ± 0.30	0.31 ± 0.06	1.79 ± 0.09	2.23 ± 0.26	0.20 ± 0.04	6.82 ± 0.47	3.82 ± 0.47	4.05 ± 0.33
Sens/OVA/LPS	6	7.07 ± 0.92‡‖	5.81 ± 0.96§	0.55 ± 0.04†§	0.71 ± 0.11†§	0§	5.65 ± 0.37	5.15 ± 0.52	5.79 ± 0.18†§

*Inflammatory response to OVA in sensitized animals exposed to LPS 50 mg/ml (or saline) 18 h after allergen challenge. Total and differential inflammatory cell counts and microvascular leakage (assessed by Evans Blue dye) were studied using the BALF. IgE and IgG levels were monitored from serum. Results are expressed as means ± SEM.

‖$P < 0.05$ and †$P < 0.01$ versus sens/sal/LPS.

‡$P < 0.05$ and §$P < 0.01$ versus sens/OVA/sal.

Influence of LPS Exposure on Primary Allergen Sensitization

Exposure of naive animals to 50 μg · ml^{-1} LPS 24 h before the sensitization (on Day 1) inhibited their allergen-induced increase in OVA-specific serum IgE levels ($P < 0.01$, $n = 6$; LPS-1 group in Table 4). OVA-specific serum IgG was significantly increased ($P < 0.01$). Challenge of these animals with OVA on Day 11 did not result in the allergen-induced inflammatory cell influx previously reported in sensitized animals 24 h after OVA challenge (Table 1). The total inflammatory cell count and the concentration of Evans Blue in their BALF were similar to those of sensitized and saline-challenged animals (the sens/sal group; Table 4) and were not significantly different from those of naive animals (Table 1). A similar profile of response was obtained in the group of animals that was exposed to LPS 1, 2, or 4 d after intraperitoneal injection with allergen (Table 4). Exposure of LPS 24 h before sensitization (the LPS/sens/OVA group) resulted in complete inhibition of allergen-induced hyperresponsiveness to MCh (PC_{150} 11.42 ± 3.17 mg · ml^{-1}), these responses being similar to that of the sensitized and saline-challenged (sens/sal) group (Figure 4).

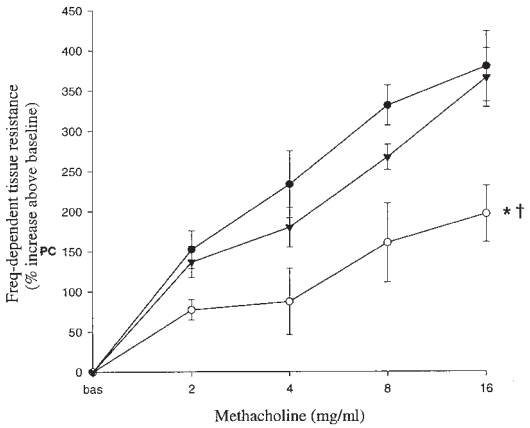


Figure 3. Effects of LPS 18 h after OVA challenge in sensitized animals. Cumulative dose–response curves to inhaled MCh (2 to 16 mg · ml^{-1}) in sensitized and OVA-challenged animals exposed to 50 μg · kg^{-1} LPS (sens/OVA/LPS) (*open circles*) ($n = 5$) or saline (sens/OVA/sal) (*inverted triangles*) ($n = 6$) 18 h after OVA challenge. Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. *$P < 0.05$ versus sens/sal/LPS (*filled circles*) ($n = 6$); that is, sensitized animals exposed to LPS 18 h after saline challenge as opposed to OVA and †$P < 0.05$ versus sens/OVA/sal.

Exposure of rats to a single aerosol challenge of LPS 6 d after intraperitoneal allergen injection did not alter the serum antibody levels in sensitized animals (Table 4). However, dramatic changes were seen in the inflammatory response measured after OVA challenge. LPS exposure on Day 6 (LPS6) further potentiated the allergen-induced cellular influx into BALF ($P < 0.01$, $n = 5$) in sensitized animals, predominantly as a result of a 25-fold increase in neutrophil influx ($P < 0.01$). Eosinophil numbers were increased 3-fold ($P < 0.01$) whereas both macrophage ($P < 0.01$) and lymphocyte ($P < 0.01$) numbers were reduced (Table 4). Allergen-induced Evans Blue leakage was further potentiated ($P < 0.01$). The pattern of inflammatory response and cellular content of BALF, leakage of Evans Blue dye, and serum OVA–specific IgE and IgG levels obtained from animals exposed to LPS on Day 8 or on Day 10 after sensitization was similar to Day 6 results (Table 4).

Histologic Analysis

Histologic assessment of parenchymal sections has shown our BAL results to closely mimic the lung inflammatory response to LPS or allergen exposure. At 24 h after allergen challenge in sensitized animals, parenchymal total inflammatory cell count was significantly increased ($P < 0.01$, $n = 4$; Figure 4, *top panel*), once again as a result of increased numbers of eosinophils ($P < 0.01$; Figure 4, *middle panel*), macrophages ($P < 0.01$), lymphocytes ($P < 0.01$), and neutrophils ($P < 0.05$; Figure 4, *lower panel*) in both tissue and alveolar spaces. LPS exposure in naive animals has been shown to induce a similar increase in cellular influx ($P < 0.01$; Figure 5) and as the lavage results have shown, this is predominantly due to neutrophil influx into the lungs ($P < 0.01$, $n = 4$; Figure 5). Eosinophils were not detected in these animals (Figure 5).

Exposure of sensitized animals to LPS 18 h after allergen challenge resulted in further exacerbation of allergen-induced inflammatory cell influx ($P < 0.05$, $n = 4$; Figure 5) and is in agreement with our BAL data. Similar exacerbated neutrophil influx ($P < 0.01$; Figure 5) and reduced number of macrophages are seen. However, LPS has further potentiated the allergen-induced eosinophil influx

TABLE 4

*Effects on inflammatory cell count, microvascular leakage, and serum antibody levels**

Exposure (*d*)	*n*	Total Count (10^6/*ml*)	Evans Blue (*mg/ml*)	Neutrophils (10^6/*ml*)	Macrophages (10^6/*ml*)	Eosinophils (10^6/*ml*)	Lymphocytes (10^6/*ml*)	OVA-Specific IgE ($1/\log_2$ *titer*)	OVA-Specific IgG ($1/\log_2$ *titer*)
Sens/sal	8	0.64 ± 0.14	1.95 ± 0.02	0.02 ± 0.01	0.44 ± 0.02	0	0.18 ± 0.04	7.32 ± 0.29	4.32 ± 0.43
Sens/OVA	11	4.16 ± 0.19**	4.36 ± 0.21**	0.26 ± 0.03**	1.59 ± 0.10**	0.16 ± 0.03**	2.15 ± 0.15**	7.03 ± 0.35	4.15 ± 0.40
LPS-1	6	0.75 ± 0.05‡	2.68 ± 0.76‡	0.08 ± 0.02‡	0.40 ± 0.08‡	0‡	0.27 ± 0.03‡	3.49 ± 0.52‡	10.16 ± 0.34‡
LPS1	5	0.90 ± 0.13‡	2.88 ± 0.51‡	0.07 ± 0.02‡	0.49 ± 0.08‡	0‡	0.33 ± 0.07‡	3.92 ± 0.57‡	9.72 ± 0.57‡
LPS2	5	1.04 ± 0.20‡	3.31 ± 0.74‡	0.10 ± 0.03†	0.49 ± 0.08‡	0‡	0.48 ± 0.15‡	2.92 ± 0.45‡	8.72 ± 0.91‡
LPS4	5	0.88 ± 0.20‡	2.88 ± 0.57‡	0.12 ± 0.02†	0.50 ± 0.06‡	0‡	0.35 ± 0.17‡	4.52 ± 0.74‡	9.12 ± 1.19‡
LPS6	5	8.36 ± 0.67‡	6.98 ± 0.34‡	6.48 ± 0.8‡	0.62 ± 0.10‡	0.43 ± 0.08‡	0.83 ± 0.23‡	7.32 ± 0.48	3.92 ± 0.55
LPS8	5	8.86 ± 0.52‡	6.23 ± 0.67‡	6.70 ± 0.51‡	0.74 ± 0.21‡	0.54 ± 0.18‡	0.88 ± 0.12‡	7.92 ± 0.67	2.92 ± 0.45
LPS10	5	8.16 ± 0.55‡	5.74 ± 0.38‡	6.23 ± 0.52‡	0.67 ± 0.22‡	0.60 ± 0.16‡	0.66 ± 0.18‡	8.32 ± 0.61	3.32 ± 0.50

*Inflammatory response in sensitized and OVA-challenged animals exposed to LPS 24 h before sensitization (LPS-1 group) or 1, 2, 4, 6, 8 and 10 d after sensitization. Serum OVA–specific IgE and IgG were measured. Total and differential cell counts in the BALF and Evans Blue leakage were studied. Results are expressed as means ± SEM.

**$P < 0.01$ versus sens/sal.

†$P < 0.05$ and ‡$P < 0.01$ versus sens/OVA.

into the lung parenchyma ($P < 0.01$, $n = 4$; Figure 5), which was not shown by the lavage results (Table 3).

Discussion

The aim of the present study was to determine the influence of bacterial products on allergen-mediated late-phase events in sensitized animals and on primary allergen sensitization *in vivo*. We characterized allergic inflammation per se in sensitized PVG rats as being associated with elevated serum OVA–specific IgE antibody, with infiltration of inflammatory cells including eosinophils into the parenchyma and BALF, as well as increased microvascular permeability and hyperresponsiveness to inhaled MCh 24 h after allergen challenge. These findings are in agreement with previous reports (20, 21). In a separate group of animals we also showed that bacterial inflammation, mimicked by exposure to aerosolized LPS, results in increased total cell count in the lavage, predominantly as a result of neutrophil influx which peaked at 6 h after exposure and remained elevated above controls for up to 24 h. Similar inflammatory response is evident in the lung parenchyma after exposure to LPS. LPS-induced Evans Blue leakage and hyperresponsiveness were also seen 6 h after exposure but were no longer apparent after 24 h. These findings are in agreement with those published by Pauwels and coworkers (22).

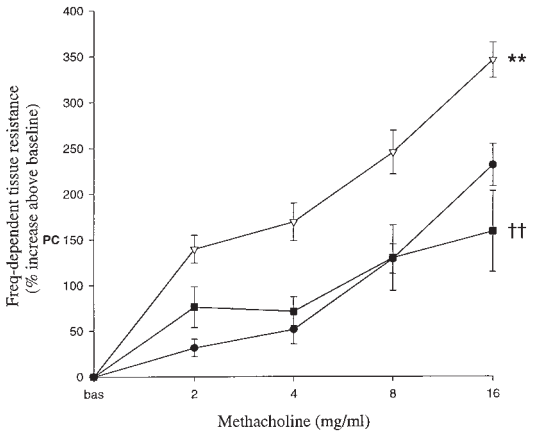


Figure 4. Effects of LPS given before sensitization. Cumulative dose–response curves to inhaled MCh (2 to 16 mg · ml^{-1}) in sensitized and OVA-challenged animals exposed to 50 μg · kg^{-1} LPS 24 h before sensitization (LPS/sens/OVA) (*filled squares*) ($n = 4$). Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. ††$P < 0.01$ versus sens/OVA (*inverted triangles*) ($n = 11$) and **$P < 0.01$ versus sens/sal (*filled circles*) ($n = 10$).

The effects of exposure to bacterial LPS on the sensitization per se can be seen in Table 4. These results clearly illustrate that if animals are exposed to LPS 1 d before and up to 4 d after sensitization with OVA, LPS inhibits the increase in OVA-specific IgE and upregulates serum IgG levels. As a consequence, no cellular influx or increased Evans Blue leakage into BALF after allergen challenge was evident, and in these animals no hyperresponsiveness to inhaled MCh was evident. When animals were exposed to LPS from Day 6 to Day 10 after sensitization a decreased OVA-specific IgG antibody level and increased serum OVA–specific IgE was seen at Day 12 compared with sensitized animals not exposed to LPS. In these sensitized animals LPS also exacerbated the allergic response to OVA, further exaggerating the Evans Blue leakage and cellular influx, in particular neutrophils and eosinophils. Studies by Michel and coworkers (23) similarly showed that LPS in house dust exacerbates symptoms in atopic asthmatics.

LPS exposure upregulates the production of Th1 cytokines, especially IFN-γ in human T cells (24), and inhibits the expression of Th2 cytokines *in vivo* (25). IFN-γ inhibits the clonal expansion of Th2 cells and suppresses IgE production by human lymphocytes during primary sensitization (26). In our animals exposed to LPS from 1 d before and up to and including 4 d after sensitization, IFN-γ would be expected to inhibit expansion of Th2 cells and

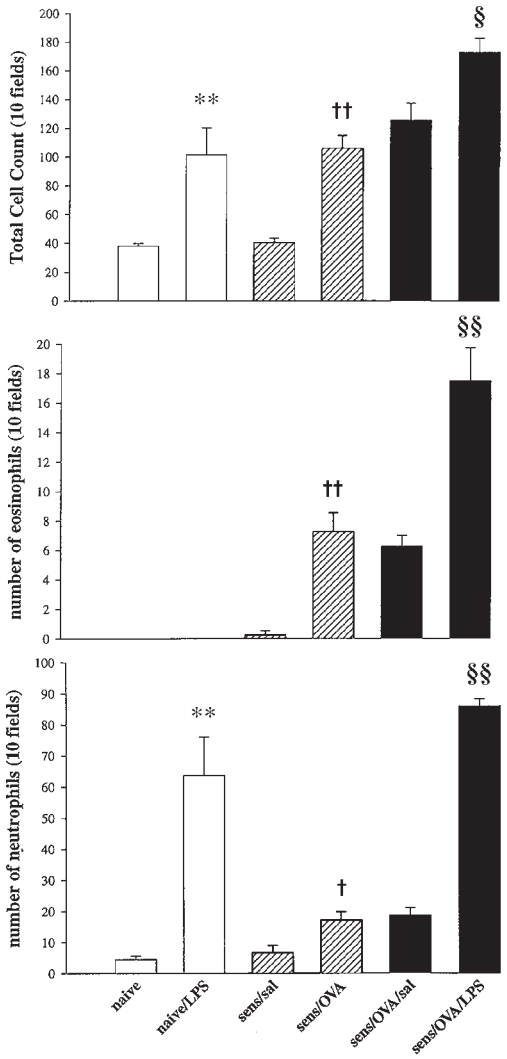


Figure 5. Histologic assessment of lung parenchyma. Effects of LPS 6 h after exposure in naive animals (*open bars*) ($n = 4$), of OVA in sensitized animals (*striped bars*) ($n = 4$), and of LPS in sensitized and OVA-challenged animals (*filled bars*) ($n = 4$) on total inflammatory cell count (*top panel*) and eosinophil (*middle panel*) and neutrophil (*lower panel*) numbers. Results are expressed as means ± SEM of 10 random, nonoverlapping lung parenchymal fields. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus sens/sal; $^{**}P < 0.01$ versus naive; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ versus sens/OVA/sal.

the synthesis of OVA-specific IgE. After the fourth day after sensitization a different process is likely to be occurring. In the primary allergic response the OVA allergen is presented to $CD4^+$ T lymphocytes largely by dendritic cells (27), resulting in the expansion of OVA-specific Th2 cell clones. These cells produce Th2 cytokines including IL-4 which, in turn, acts on "virgin" B cells to produce memory B cells and IL-4–independent, IgE-producing plasma cells that are no longer sensitive to the inhibitory effect of IFN-γ (28). Our data would suggest that in the first 4 d after sensitization, IFN-γ and/or IL-12 produced by LPS exposure drives the B cells toward IgG antibody production but inhibits class switching through to IgE. The different pattern of response to LPS seen when 6 or more d are allowed to lapse between primary sensitization and LPS exposure suggests that plasma cells committed to producing IgE are present by Day 6, and these committed IgE-producing cells are resistant to IL-12 and/or IFN-γ produced in response to LPS exposure. LPS must be present before the isotype switch to IgE has occurred by Day 6 to exert its inhibitory effects.

In this paper we report that LPS induced hyperresponsiveness in naive as well as in sensitized but saline-challenged animals. The mechanisms involved in this increased responsiveness to MCh is currently unknown, however a recent report by Andersson and coworkers at an International Meeting of the American Thoracic Society (29) suggests it may be the downregulation of the constitutive nitric oxide synthase (cNOS) isoenzyme that is responsible for this phenomena. These researchers have shown LPS to halve the cNOS activity in the rat lung and the trachea 6 h after intratracheal instillation with LPS. Similarly, data reported by Schuiling and colleagues further point to an involvement of decreased levels of cNOS-derived nitric oxide in the airway hyperreactivity seen after allergen challenge in sensitized guinea pigs (30).

Exposure to LPS 18 h after OVA challenge in sensitized animals further exacerbated the cellular allergic inflammatory response to the allergen. The profile of the inflammatory response was characterized by exaggerated neutrophil influx and microvascular leakage without eosinophil influx into the BALF. In addition, the usual postallergen challenge hyperresponsiveness to inhaled MCh was abolished. This reported dissociation between neutrophil influx and airway hyperresponsiveness has previously been shown in our laboratory using Brown–Norway rats (unpublished observation) and is in agreement with reports of ozone-induced lung inflammation (31, 32).

The mechanism by which LPS is able to normalize responses to MCh in allergic animals may be explained by its stimulation of inhibitory cytokines IL-12 and/or IFN-γ. IL-12 is produced by monocytes and dendritic cells in response to bacterial products, including LPS. In the presence of IL-4, LPS stimulates the production of high levels of IL-12 by human dendritic cells *in vitro* compared with low levels in the absence of IL-4 (33). Schwarze and coworkers have shown IL-12 to inhibit airway hyperresponsiveness to MCh after OVA exposure in sensitized mice (34). This mechanism may be responsible for the lack of hyperresponsiveness observed in our sensitized and allergen-challenged animals exposed to LPS. Further, Schwarze and coworkers suggested that LPS may serve to prevent the influx of eosinophils into the BALF after allergen challenge. This is supported by our results in sensitized animals exposed to LPS 18 h after OVA challenge, where we failed to detect eosinophils in the BALF even though histology has shown eosinophil accumulation in the lung parenchyma, suggesting a possible LPS effect on regulation of eosinophil chemoattractants and/or eosinophil apoptosis. The presence of LPS in aerosolized OVA (given chronically) has been shown to prevent OVA-induced eosinophilia in guinea pigs (35), and contamination of allergen used for bronchial challenge with LPS resulted in alteration of cellular inflammation (36).

To summarize, in this study we have demonstrated that in the PVG rat, exposure to the bacterial product LPS has the ability to prevent sensitization to allergen *in vivo* only if the exposure occurs early in the sensitization process, which in this model means up to Day 6 after primary sensitization with allergen. Exposure to LPS after Day 6 further aggravated the allergic inflammatory response. In our study LPS can thus be seen as having dual effects. First, exposure to LPS after allergen challenge in sensitized animals inhibited MCh hyperresponsiveness and eosinophil influx into the BALF which we can propose to be associated with its stimulation of high levels of IL-12 resulting in inhibition of Th2-driven allergic response (34). LPS also stimulated B-cell activity. Up to and including 4 d after sensitization, LPS was able to stimulate the B cells to differentiate into IgG-producing plasma cells. However, if the isotype switch had already occurred, LPS then directly stimulated those B cells which have been primed to produce IgE.

It is currently accepted that respiratory viral infections are important "trigger factors" in the development of allergic respiratory diseases, particularly asthma (37, 38). Unlike viral infections, bacterial infections do not generally trigger asthma and in fact, early exposure may protect the individual from development of atopy and asthma later in life (8). In this study we have reported additional evidence suggesting a potentially protective role of bacterial exposure against primary allergen sensitization which could result in disease modification involving alterations in the natural history of asthma. This mechanism may contribute to the variations in the frequency of atopy/asthma that have recently been reported between first- and second-world countries, which point to an inverse relationship between disease prevalence and socioeconomic status (39–41). These findings have led to the development of the "hygiene" hypothesis which suggests that decreasing levels of exposure to infections (42, 43) and/or commensal microbial stimuli (6, 8) in developed countries, particularly during the induction of primary Th1/Th2 responses to aeroallergens during early life (8, 44), may be responsible for increased disease prevalence. Bacterial LPS has been suggested as a potential mediator of these effects (8), and in this context it is interesting to note that a recent study has identified a polymorphism in the gene encoding the high-affinity receptor for LPS (CD14) which is associated with atopy intensity (45).

Acknowledgments: This study was supported by the National Health and Medical Research Council, Australia, and the Asthma Foundation of Western Australia.

References

1. Djukanovic, D., W. R. Roche, J. W. Wilson, C. R. W. Beasley, P. Twentyman, P. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* 142:434–457.
2. Larsen, G. L., R. A. Bethel, C. G. Irvin, R. J. Martin, and D. A. Uchida. 1991. Granulocytes and airway reactivity. *Am. Rev. Respir. Dis.* 143:S64–S65.
3. Bentley, A. M., G. Menz, C. H. R. Storz, D. S. Robinson, B. Bradley, P. K. Jeffery, S. R. Durham, and A. B. Kay. 1990. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. *Am. Rev. Respir. Dis.* 146:500–506.
4. Sunyer, J., J. M. Anto, J. Castellsague, J. B. Soriano, and J. Roca. 1996. Total serum IgE is associated with asthma independently of specific IgE levels. *Eur. Respir. J.* 9:1880–1884.
5. Bjorksten, B. 1996. Immunological outcome measures. *Eur. Respir. J.* S21: 22s–27s.
6. Holt, P. G. 1995. Environmental factors and primary T-cell sensitization to inhalant allergens in infancy: reappraisal of the role of infections and air pollution (Review). *Pediatr. Allergy Immunol.* 6:1–10.
7. Bjorksten, B. 1994. Risk factors in early childhood for the development of atopic diseases. *Allergy* 49:400–407.
8. Holt, P. G., P. D. Sly, and B. Bjorksten. 1997. Atopic versus infectious diseases in childhood: a question of balance? *Pediatr. Allergy Immunol.* 8:53–58.
9. Peat, J. K. 1996. Prevention of asthma. *Eur. Respir. J.* 9:1545–1555.
10. Johnston, S. L., P. K. Pattemore, G. Sanderson, S. Smith, F. Lampe, L. Josephs, P. Symington, S. O'Toole, S. H. Myint, D. A. Tyrrell, and S. T. Holgate. 1995. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ (Clinical Research Ed.)* 310:1225–1229.
11. Bjornsson, E., E. Hjelm, C. Janson, E. Fridell, and G. Boman. 1996. Serelogy of *Chlamydia* in relation to asthma and bronchial hyperresponsiveness. *Scand. J. Infect. Dis.* 28:63–69.
12. Yano, T., Y. Ichikawa, S. Komatu, S. Arai, and K. Oizumi. 1994. Association of *Mycoplasma pneumoniae* antigen with initial onset of bronchial asthma. *Am. J. Respir. Crit. Care Med.* 149:1348–1353.
13. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties (Review). *Annu. Rev. Immunol.* 7:145–173.
14. Barnes, P. J., and F. Y. Liew. 1995. Nitric oxide and asthmatic inflammation. *Immunol. Today* 16:128–130.
15. Borish, L., and L. Rosenwasser. 1997. TH1/TH2 lymphocytes: doubt some more. *J. Allergy Clin. Immunol.* 99:161–164.
16. Peták, F., Z. Hantos, Á. Adamicza, T. Asztalos, and P. D. Sly. 1997. Methacholine-induced bronchoconstriction in rats: effects of intravenous vs. aerosol delivery. *J. Appl. Physiol.* 82:1479–1487.
17. Hantos, Z., A. Adamicza, E. Govaerts, and B. Doroczy. 1992. Mechanical impedances of lungs and chest wall in the cat. *J. Appl. Physiol.* 73:427–433.
18. Van Halteren, A. G. S., M. J. F. Van Der Cammen, J. Biewenga, H. F. J. Savelkoul, and G. Kraal. 1997. IgE and mast cell responses on intestinal allergen exposure: a murine model to study the onset of food allergy. *J. Allergy Clin. Immunol.* 99:94–99.
19. Sedgwick, J. D., and P. G. Holt. 1983. Induction of IgE-isotype specific tolerance by passive stimulation of the respiratory mucosa. *Immunology* 50: 625–630.
20. Elwood, W., J. O. Lotvall, P. J. Barnes, and K. F. Chung. 1991. Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized Brown-Norway rats. *J. Allergy Clin. Immunol.* 88:951–960.
21. Elwood, W., P. J. Barnes, and K. F. Chung. 1992. Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic Brown-Norway rats. *Int. Arch. Allergy Immunol.* 99:91–97.
22. Pauwels, R. A., J. C. Kips, R. A. Peleman, and M. E. Van der Straeten. 1990. The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. *Am. Rev. Respir. Dis.* 141:540–545.
23. Michel, O., R. Ginanni, B. Le Bon, J. Content, J. Duchateau, and R. Sergysels. 1992. Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am. Rev. Respir. Dis.* 146:352–357.
24. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170: 827–846.
25. Morris, S. C., K. B. Madden, J. J. Adamovicz, W. C. Gause, B. R. Hubbard, M. K. Gately, and F. D. Finkelman. 1994. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. *J. Immunol.* 152:1047–1056.
26. Pene, J., F. Rousset, F. Briere, I. Chretien, J. Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. I. Arai, J. Banchereau, and J. E. De Vries. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2. *Proc. Natl. Acad. Sci. USA* 85:6880–6884.
27. Kay, A. B. 1991. Lymphocytes in asthma (Review). *Respir. Med.* 85:87–90.
28. Banchereau, J., F. Bazan, D. Blanchard, F. Briere, J. P. Galizzi, C. Van Kooten, Y. J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922.
29. Andersson, D., A. Miller-Larsson, and R. Brattsand. 1998. Budesonide prevents inflammation-induced changes in nitric oxide synthase activities (cNOS and iNOS) in rat lung. *Am. J. Respir. Crit. Care Med.* 175:A823. (Abstr.)
30. Schuiling, M., A. B. Zuidhof, M. A. A. Bonouvrie, N. Venema, J. Zaagsma, and H. Meurs. 1998. Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. *Br. J. Pharmacol.* 123:1450–1456.
31. Kato, H., M. Salmon, B. Haddad, T. J. Huang, J. Zagorski, and K. F. Chung. 1997. Role of cytokine-induced neutrophil chemoattractant (CINC) in ozone-induced airway inflammation and hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 156:234–239.
32. Evans, T. W., J. J. Brokaw, K. F. Chung, J. A. Nadel, and D. M. McDonald. 1988. Ozone-induced bronchial hyperresponsiveness in the rat is not accompanied by neutrophil influx or increased vascular permeability in the trachea. *Am. Rev. Respir. Dis.* 138:140–144.

33. Verhasselt, V., C. Buelens, F. Willems, D. De Groote, N. Haeffner-Cavaillon, and M. Goldman. 1997. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *J. Immunol.* 158:2919–2925.
34. Schwarze, J., E. Hamelmann, G. Cieslewicz, A. Tomkinson, A. Joetham, K. Bradley, and E. W. Gelfand. 1998. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. *J. Allergy Clin. Immunol.* 120:86–93.
35. Rylander, R., and P. G. Holt. 1998. Modulation of immune response to inhaled allergen by co-exposure to the microbial cell wall components (1-3)-β-D-glucan and endotoxin. *Mediators in Inflammation* 7:105–110.
36. Hunt, L. W., G. J. Gleich, T. Ohnishi, D. A. Weiler, E. S. Mansfield, H. Kita, and S. Sur. 1994. Endotoxin contamination causes neutrophilia following pulmonary allergen challenge. *Am. J. Respir. Crit. Care Med.* 149: 1471–1475.
37. Busse, W. W. 1989. The relationship between viral infections and onset of allergic diseases and asthma (Review). *Clin. Exp. Allergy* 19:1–9.
38. Busse, W. W. 1994. The role of respiratory infections in airway hyperresponsiveness and asthma. *Am. J. Respir. Crit. Care Med.* 150:S77–S79.
39. Von Mutius, E., F. D. Martinez, C. Fritzsch, T. Nicolai, G. Roell, and H. H. Thiemann. 1994. Prevalence of asthma and atopy in two areas of West and East Germany. *Am. J. Respir. Crit. Care Med.* 149:365–370.
40. Strachan, D. 1996. Socioeconomic factors and the development of allergy (Review). *Toxicol. Lett.* 86:199–203.
41. Willies-Jacobo, L. J., J. M. Denson-Lino, A. Rosas, R. D. O'Connor, and N. W. Wilson. 1993. Socioeconomic status and allergy in children with asthma. *J. Allergy Clin. Immunol.* 92:630–632.
42. Strachan, D. P. 1989. Hay fever, hygiene, and household size. *Br. Med. J.* 299:1259–1260.
43. Martinez, F. D. 1994. Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? (Review). *Thorax* 49:1189–1191.
44. Holt, P. G., and C. Macaubas. 1997. Development of long-term tolerance versus sensitization to environmental allergens during the perinatal period (Review). *Curr. Opin. Immunol.* 9:782–787.
45. Baldini, M., I. C. Lohman, M. Halonen, R. P. Erickson, P. G. Holt, and F. D. Martinez. 1999. A polymorphism in the 5′-flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am. J. Respir. Cell Mol. Biol.* 20:976–983.

Antibodies against molds and mycotoxins following exposure to toxigenic fungi in a water-damaged building

Aristo Vojdani

MOLDS represent a subgroup of fungi, which can grow where moist conditions exist (e.g., on wood, leather, carpets, clothing, drywall, sheet rock, and insulation). Given that molds grow in wet or moist indoor environments, individuals are sometimes exposed to molds or to their byproducts. Such exposures can occur either by direct contact with surfaces, or through the air if mold byproducts are aerosolized. (1) Exposure to certain types of airborne molds and their spores can cause allergic reactions, episodes of asthma, and other respiratory problems in individuals who are predisposed genetically and immunologically.

Health problems that result from molds may also occur when individuals are exposed to large doses of chemicals called "mycotoxins," which are produced by molds. (2-4) Mycotoxins are fungal metabolites that exert toxic effects ranging from severe irritation to immunosuppression and cancer. Virtually all of the information related to disease induced by mycotoxins concerns ingestion of contaminated food. (5) In heavily contaminated environments, neurotoxic symptoms related to airborne mycotoxin exposure have been noted. (6)

Several species of molds, including some found indoors in contaminated buildings, produce mycotoxins. (7-9) During the past 10 yr, concern has escalated about exposure to multiple mycotoxins from a mixture of mold spores growing in wet indoor environments. (10-12) Adverse health effects from exposure to a mixture of mold spores are different from those related to a single mycotoxin exposure under controlled conditions. Indoor exposure to molds and their spores or mycotoxins more closely resembles the field exposure of animals to the entire mold or its byproducts than it does the controlled experimental laboratory exposure to a single agent via a single exposure route. (13-14)

In contrast to a controlled laboratory exposure, field exposure of animals to molds produces effects on the immune system which are manifested as increased susceptibility to infectious diseases. (13) Many mycotoxins are also cytotoxic; therefore, depending on the route of entry, mycotoxins may be damaging to the skin, lung, or gut. Such cytotoxicity to the lungs may (a) affect the physical defense mechanisms of the respiratory tract; (b) damage alveolar macrophages; and (c) decrease the airway's ability to clear bacteria, viruses, and other particulate matter in humans. (13) The combined outcome of these immunosuppressive and cytotoxic activities of mycotoxins increases the susceptibility of the exposed person to infectious diseases, and possibly cancer.

Several toxigenic molds have been found by different laboratories during air-quality investigations of damp homes and offices. Among the genera found most frequently in numbers that exceed levels found outdoors are Aspergillus, Penicillium, Stachybotrys, Chaetomium, Alternaria, and Cladosporium. (14-18) However, systematic studies of health effects and immunological reactions related to indoor exposure to these molds, and antibody production against their antigens and toxins, have not been studied simultaneously. Johanning et al. (17) used Stachybotrys extracts and enzyme-linked immunosorbent assay (ELISA) and radioallergosorbent (RAST) protocols to measure mold-specific immunoglobulin (Ig)G and IgE in the blood of individuals exposed to toxigenic fungi. They found no elevated levels of these antibodies to S. chartarum, which was statistically significant. (17) In another study, an increase in IgA production and IgA nephropathy was reported in mouse experiments after the rodents were injected with trichothecene of vomitoxin. (19) Similar findings were reported when IgE, IgG, and IgA antibodies against S. chartarum were measured in patients with asthmatic or mycotoxicosis symptoms. Mycelium extract was used in the ELISA assay, and the Stachybotrys-specific IgG and IgA were detected in the group of exposed subjects--but not among the control groups. IgA and IgG levels were significantly higher ($p < 0.01$ and $p < 0.05$, respectively) in the patient group than in the control group. The IgE levels did not differ between groups. Raunio et al. (20) suggested that

exposure to Stachybotrys did not cause IgE-mediated allergy in humans, and that the IgA response better reflected exposure to the fungus than did the IgG response.

Other investigators have reported that the serum IgA level was a more specific factor than IgG level for indicating Farmer's Lung disease, which is also associated with fungal exposure. Investigators reached the same conclusion concerning antibodies against Aspergillus fumigatus. (21,22) When specific IgA was detected in a patient's sera, the IgA concentrations in bronchoalveolar lavage were high. These results suggest that the concentration of serum IgA may depend on the magnitude of respiratory exposure to fungi. (23)

Given that the respiratory tract is the major route of exposure for fungi, their spores, and mycotoxins, we conducted the present study to measure specific IgG, IgM, IgE, and IgA in blood against 7 different molds found most frequently in water-damaged buildings. Moreover, we analyzed these antibodies against molds, as well as against 2 of the most common mycotoxins they produce, to develop a more useful biomarker of exposure in the work environment. Simultaneous detection of antibodies against both toxigenic fungi and their mycotoxins may indicate occupational exposure to molds and their metabolites in a subpopulation of individuals exposed to molds.

Method and Materials

Environmental exposure evaluation and patients. The study population of 40 patients (e.g., executives, directors, secretaries, and administrators [among others], who ranged in age from 26 yr to 62 yr of age [11 males and 29 females]) worked for various organizations and was exposed occupationally to molds under the same indoor environmental conditions. Most subjects had moved to offices in the building in 1995 or earlier. In December 1998, major flooding occurred, and water entered the roof of the building. The flood scenario was described as "waterfalls from the ceiling"; the building and its fittings became damp, moist, and broken. Carpets felt "spongy" when walked upon. Attempts were made to patch the roof; however, it continued to leak during the year 2000. Commencing in the summer of 2001, patients could see colorful molds growing on the walls, and portions of the ceiling began to peel. The musty odor that pervaded the building was so unpleasant that the employees chose to wear paper masks. Soon after these events occurred, employees began to seek medical help.

Each employee was administered a health survey--during which a questionnaire developed by the American College of Occupational and Environmental Medicine (Arlington Heights, Illinois) was administered and a complete physical examination was conducted. Neurological and behavioral symptoms--including blurred vision, memory loss, fatigue, headache, migraine headache, nausea, loss of balance, cognitive deficits, rhinitis, sinusitis, nosebleeds, rashes, allergies, and painful lymph nodes--were recorded. Reported symptoms were similar to those indicated by patients with Chronic Fatigue and Fibromyalgia syndromes. Some of these symptoms were confirmed by pulmonary function testing and single-photon emission computed tomography (SPECT) scan. Environmental engineering firms tested the building (i.e., tape transfer, swab samples, and viable microbial activities were performed at different locations, including walls, ceilings, tiles, and sinks), and elevated levels (i.e., > 2,000 CFU/swab) of Aspergillus, Alternaria, Cladosporium, Penicillium, Stachybotrys, and Chaetomium were reported. Blood samples were drawn from the patients and from 40 age- and sex-matched control subjects who had no history of exposure to toxigenic indoor molds or symptoms associated with such exposure. The samples were sent to our laboratory by overnight courier, maintained at 4[degrees]C, and subjected to various examinations (described later).

All patients gave their informed consent and allowed inclusion of their data in this manuscript without disclosure of their identity in the publication. Furthermore, we removed the specimens' identifier numbers from the samples, labeled them 1-40, and maintained them at -20[degrees]C. A few months later, levels of IgG, IgM, IgA, and IgE antibodies against 9 different mold antigens were measured. Inasmuch as residual serum material was examined retrospectively, and at the time of examination samples did not have patient/donor identifiers, this study was exempt from oversight by the institutional review board.

Preparation of fungal antigen. The protocol for optimal fungal antigen extract preparation was followed, using procedures described previously. (24-27) Molds, Stachybotrys chartarum, Chaetomium globosum, Penicillium notatum, Aspergillus niger, Alternaria alternata, Cladosporium herbarum, and Epicoccum nigrum were obtained from the American Type Culture Collection (Rockville, Maryland). Initially, the molds were cultured on 2% malt extract agar (MEA) for 8 days at 25[degrees]C, after which spore suspensions were prepared in 0.1 M phosphate buffered saline (PBS) (pH 7.4) that contained 0.05% Tween 20[TM] (i.e., sorbitan mono-9-octadecenoate poly[oxy-1,1-ethanedyl]). For each spore suspension, 1 ml was inoculated into 100 ml of 2% malt extract broth (for Stachybotrys), or into cellulose broth (for the others) in untreated glass bottles, and the cultures were incubated for 10 days at 25[degrees]C. Mycelium was separated from the broth by centrifugation at 2,000 g for 20 min, dried in a vacuum dryer, and stored at -70[degrees]C. Dried mycelium-containing spores were suspended at 50 mg/ml in 0.1 M PBS (pH 7.5) containing 0 02% phenylmethyl sulfonyl fluoride (PSF) and 0.02% sodium azide. The mycelium suspension was sonicated for 5 sec at an output of 70% with Virsonic 50 (Virtis Company [Gardiner, New York]). The sonication step was repeated 10 times for maximum cell disruption; thereafter the suspension remained on a shaker for 24 hr at 4[degrees]C. Following centrifugation at 4,000 g, the supernatant was dialyzed at molecular cut-off 2kD against PBS at 4 [degrees]C for 24 hr, lyophilized, and stored at -70[degrees]C. For quality control and reproduction of antigenic preparation of these mold extracts, 20 mg of each was dissolved in 1 ml of 0.01 M PBS, the protein content was determined, (28) and its components were analyzed by 15% sodium dodecyl sulfate (SDS) gel electrophoresis (Bio-Rad Laboratories [Hercules, California]).

Preparation of mycotoxins. Mycotoxins from different molds were prepared in accordance with a modified method that originated in our laboratory. (17) A total of 100 mg of dried mycelium was extracted with 2 ml of 20% methanol in chloroform at 40[degrees]C, with repeated sonication for 30 min. The extract was passed through a silica gel column (Bio-Rad Laboratories [Hercules, California]) and washed with 10 ml of 8% methanol in dichloromethane. The eluent was evaporated under a stream of dry nitrogen, and the remaining oily material was dissolved in 1 ml of ethanol and analyzed by reversed-phase high-performance liquid chromatography (HPLC) (ESA [Chelmsford, Massachusetts], model 5600 Coularray Detector, with solvent delivery pump model 580 and an analytical cell that uses 2 porous graphite electrodes). The column was C-18 rainin 5-[micro]m 4.6 x 250 mm, with a 15-min gradient of 60-75% methanol in water, a flow rate of 1 ml/min, and monitoring at 260 nm. Two peaks--1 at 10 6 min and the other at 12.2 min--were obtained which corresponded in retention time to those of satratoxin H and trichothecene. The total amounts of satratoxin H and trichothecene were obtained in 100 mg of sample and were estimated at approximately 1 5 [micro]g and 1.7 [micro]g, respectively.

Binding of mycotoxins to human serum albumin (HSA). Satratoxin was coupled to the carrier protein HSA with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-4-toluolsufonate (CCMT) and with succinic anhydride. A total of 100 [micro]g of satratoxin in 100 [micro]l pyridine was reacted with 2 mg of succinic anhydride in a 45[degrees]C water bath with off-and-on vortexing. The reaction mixture was evaporated to dryness. The satratoxin-hemisuccinate was dissolved in 200 [micro]p of dimethyl formamide and was added dropwise to a 1-ml solution of 0.1 M carbonate buffer (pH 9.5) that contained 10 mg of HSA and 2 mg of CCMT. The mixture was kept on the stirrer for 4 hr, followed by the addition of 1 mg CCMT and adjustment of the pH to 7.5 with 1 M HCI, followed by an additional incubation of 4 hr. Finally, after the addition of 1 mg of CCMT, the pH was adjusted to 5.5 and the mixture was kept on the stirrer for 4 hr at room temperature. Uncoupled residues of the reagents and derivatives were removed by dialysis at a cutoff of 2,000 g against 0.1 M PBS (pH 7.2) for 48 hr. Following centrifugation at 10,000 g, binding of satratoxin to HSA was examined by SDS gel electrophoresis. A shift in the location of the HSA band after the addition of mycotoxin was used as evidence for the binding capacity of satratoxin to the carrier protein.

ELISA for detection of IgG, IgM, IgA, and IgE against fungal antigens and mycotoxins We analyzed levels of IgG, IgM, IgA, and IgE against antigens of molds in human sera by indirect ELISA. Microtiter plates were coated with 0 1 ml of HSA in duplicate, to serve as controls, or with mold extract and mycotoxins at a protein concentration of 10 [micro]g/ml. Following incubation, washing, and blocking with 2% bovine serum albumin (BSA), 0.1 ml of human serum, at an optimal dilution of 1:2 for IgE and 1:100 in serum diluent buffer (2% BSA in 0.1 ml PBS plus 0.01% Tween 20) for IgG, IgM, and IgA, were added into

the duplicate wells of the plates. Plates were incubated at 37[degrees]C for 2 hr and washed 3 times with PBS Tween 20 (PBST), after which 0.1 ml of affinity-purified goat anti-human IgG([gamma]), IgM([mu]), IgA([alpha]), or IgE([epsilon]) light-chain-specific, conjugated with peroxidase at dilutions of 1:500, were added and incubated at 37[degrees]C for 1 hr. Following 4 washings with PBST and the addition of 0.1 ml of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate, color development was measured. After incubation for 30 min, and addition of a stop solution, the intensity of color was measured spectrophotometrically at 492 nm. For each specimen, the ELISA background reading of wells coated with HSA was automatically subtracted from the wells coated with mold antigens. The background reading of wells coated with nonspecific antigen (i.e., HSA) reacted with the serum and all other reagents and was less than 12% of the absorbances of the wells coated with mold antigens. in each assay, serially diluted sera from rabbits immunized with molds antigens, and from patients after immunotherapy with high levels of antibodies against different molds, were used for the construction of a standard curve. We plotted the mean absorbances obtained from duplicate wells from each calibrator against their optical density (OD) values to determine the ODs for unknown samples.

Coefficients of intra-assay and inter-assay variations. We calculated coefficients of intraassay variations by running 5 samples 8 times in 1 assay. Coefficients of interassay variations were determined by measuring the same samples in 6 consecutive assays. This replicate testing (a) established the validity of the ELISA assays; (b) determined the appropriate dilution with minimal background; and (c) detected serum, IgG, IgM, igA, and IgE against different antigens. Two sera from healthy controls and 2 from patients exposed to molds were used for the construction of control curves.

Determination of optimal dilution of serum. We determined the optimal dilutions of sera by diluting 20 different controls' and patients' sera 1:25-1:400 in serum diluent buffer and adding the sera to duplicate wells coated with either HSA or mold antigens. Dilutions between 1:50 and 1:200 resulted in a good linearity. Therefore, a serum dilution of 1:100 was used for all IgG, IgM, and IgA antibody assays.

For IgE assay, 20 different sera from controls and patients were used undiluted or diluted from 1:2 to 1:8 in serum diluent buffer. All other steps were similar to IgG, IgM, or IgA determinations. Good linearity was maintained throughout the IgE calibration range. However, given that a dilution of 1:2 provided minimal background, it was used in the IgE assay.

Antibody specificity testing by absorption of sera. Specificity of the ELISA assay for molds and mycotoxin antibodies was confirmed by mold antigens or mycotoxin competition. For this testing, 6 different sera with high levels of IgG, IgM, and IgA antibodies (OD in ELISA > 0.8) against Stachybotrys or satratoxin were used in different test tubes. One ml of each serum was preincubated with 1 mg of HSA, Stachybotrys antigens, satratoxin and trichothecene bound to HSA, or 10 ng of each mycotoxin alone. After mixing, tubes were kept for 1 hr in a 37[degrees]C water bath, followed by 1 hr of incubation at 4[degrees]C and centrifugation at 3,000 g for 10 min. The supernatant was used for measurement of IgG, IgM, and IgA antibody levels against Stachybotrys antigens and mycotoxins.

Statistical analysis. Systat program version 5.2 (Systat, Inc. [Evanston, Illinois]) was used for statistical analysis. Normal distribution of data was tested by the Kolmogorov-Smirnov 1-sample test. One-way analysis of variance (ANOVA) was performed with ANOVA and the Kruskal-Wallis H test for ranked data. For post hoc analysis, Tukey's test was employed. Correlations were performed with Pearson's test and the nonparametric Spearman's rank order test. In this study, p values [less than or equal to] 0.05 were considered significant.

Results

Fungal contamination in a water-damaged building. Environmental laboratory examinations of the water-damaged office building were conducted by an environmental engineering firm. Microscopic and culture analysis revealed significant fungal contamination with Alternaria, Aspergillus, Chaetomium, Cladosporium, Epicoccum, Penicillium, and Stachybotrys.

Concentration samples of these molds were taken from 7 different locations in the building. In 4 of 7 swab samples, viable microbial activity on MEA and cellulose agar exceeded 2,000 colony-forming units (CFU/swab) (Table 1). Sample 2 was dominated by Stachybotrys; sample 4 by Penicillium and Chaetornium; sample 5 by Epicoccum and Penicillium; and sample 6 by Penicillium and Epicoccum. Total bioaerosols were also measured by collecting air samples and culturing on MEA and cellulose agar. Results of 4 different samples showed a range of 42-32,251 CFU/[m.sup.3] of molds, with Cladosporium being the predominant species (Table 2). In air samples 1 and 3, the concentration of Cladosporium was approximately 1 x [10.sup.3] and 3 x [10.sup.4] CFU/[m.sup.3], respectively (Table 2). Detection of a significantly high level (> 400 CFU/[m.sup.3]) of molds strongly suggested that reservoirs of spores existed in the building at the time of sampling. Patients reported to their examining physicians the presence of a "strong musty odor." In fact, they stated that the musty odor was so offensive that they began wearing masks, which allowed them to function more effectively. Soon afterwards, they presented with neurological and behavioral symptoms. The most common symptoms were fatigue, headache, easily losing one's breath, muscle and joint pain, memory disturbance, multiple chemical sensitivities, and allergies. Some of these abnormalities were confirmed by pulmonary function tests and SPECT scans, which were abnormal in 60% and 80% of patients, respectively (Table 3). These symptoms were present in a statistically significant ($p < 0.0001$) larger proportion (55-92.5%) of employees in the contaminated office building than in controls (0-25%).

Detection of IgG, IgM, IgA, and IgE antibodies. Sera from healthy control subjects who had no history of exposure to molds, and from 40 patients exposed to molds or their spores in the indoor environment, were analyzed for the presence of IgG, IgM, IgA, and IgE antibodies against different mold antigens and mycotoxins. The ELISA results were expressed by individual and mean [+ or -] standard deviation (SD) of OD at 492 nm. The OD for IgG antibody values obtained with a 1:100 dilution of healthy control sera ranged from 0.01 to 1.27 OD and varied among subjects and mold antigens. The mean [+ or -] SD of these OD values ranged from 0.16 [+ or -] 0.14 OD to 0.24 [+ or -] 0.26 OD. The corresponding IgG OD values from mold-exposed patients' sera ranged from 0.02 to 1.97 OD; mean [+ or -] SD levels for IgG values varied from mold to mold and ranged from 0.30 [+ or -] 0.25 OD to 0.52 [+ or -] 0.53 OD. For all 7 molds and the 2 mycotoxins, the difference between mean [+ or -] SDs of controls' sera and mold-exposed patients' sera were statically significant (Alternaria: $p = 0.008$; Aspergillus, Stachybotrys, and Chaetomium: $p = 0.001$; Cladosporium: $p = 0.009$; Epicoccum: $p = 0.003$; Penicillium: $p = 0.0005$; satratoxin: $p = 0$ 013; and trichothecene: $p = 0.049$ [Figs. 1-4]).

[FIGURES 1-4 OMITTED]

At OD values above the mean of 2 standard deviations in the healthy control group, levels of IgG antibodies against these antigens were calculated in controls' and patients' sera, and we found that even though 10-15% of the controls' sera had IgG values that were 2 standard deviations higher than the mean control group, the mold- and mycotoxin-exposed group revealed an elevation in IgG values from 20% to 42.5% ($p < 0.01$ [Table 4]). The individual data presented in Figures 1-4 showed that if specimens from controls and patients presented an elevation in IgG, IgM, IgA, and IgE antibodies against 1 or more molds, they also showed a significant elevation in antibodies against mycotoxins. However, we found several specimens that were positive for IgG antibodies against 1 or more molds that did not have any antibodies against satratoxin or trichothecene. Levels of IgA antibodies against molds and mycotoxins in sera of healthy controls and mold-exposed patients are shown in Figure 2. These serum IgA antibodies against all 7 molds and the 2 mycotoxins were significantly higher in patients than in controls. The mean [+ or -] standard deviation for controls ranged from 0.17 [+ or -] 0.16 OD to 0.27 [+ or -] 0.29 OD; for patients, these values ranged from 0.35 [+ or -] 0 34 OD to 0.67 [+ or -] 0.70 OD. Differences between IgA antibody levels in controls and patients were statically significant (i.e., Alternaria: $p = 0.008$, Aspergillus: $p = 0.01$, Chaetomium: $p = 0.006$, Cladosporium: $p = 0.007$, Penicillium: $p = 0.015$, Stachybotrys: $p = 0.002$, satratoxin: $p = 0.018$, and trichothecene: $p = 0.004$. With respect to Epicoccum, however, the differences in IgA levels were not significant ($p = 0.25$). When we used 2 standard deviations of the mean control as the cutoff point, 10-17.5% of controls vs. 22.5-42.5% of mold-exposed patients showed elevated IgA antibody levels ($p < 0.01$). The highest percentage of IgA antibody levels was initially attributed to Stachybotrys--followed by Penicillium and Chaetomium (Table 3).

Likewise, IgM antibody levels against these molds and mycotoxins were examined in both groups. The mean [+ or -] standard deviation of the data showed significant differences between control and patient groups only for Stachybotrys, Cladosporium, Alternaria, Aspergillus, satratoxin, and trichothecene ($p < 0.05$)--but not for Chaetomium, Epicoccum, or Penicillium. The percentage of elevated serum IgM antibodies against these molds and mycotoxins were elevated more significantly in patients than in controls for Stachybotrys, satratoxin, Cladosporium, and Penicillium (Table 3). Similar to IgG anti-mold antibody values, IgA and IgM levels against mycotoxins were elevated only when the same specimens had high levels of antibodies against 1 or more molds.

Fungal-specific IgE antibodies were measured using similar microplates coated with fungal antigens and mycotoxins. Each serum was assayed at a 1:2 dilution in serum diluent, in duplicate The mean [+ or -] SD for IgE levels is shown in Figure 4 Whereas the overall mean [+ or -] SD for IgE values was higher in patients than in controls, only Aspergillus and satratoxin values reached a statistically significant level of p [less than or equal to] 0.05; for other molds and mycotoxins, p values exceeded 0.05. However, at cutoff values of 2 standard deviations, 7.5-12.5% of control sera and 12.5-27.5% of mold-exposed sera of patients revealed elevated IgE values (Table 4).

Specificity, intra-assay, and inter-assay precision. The coefficient of intra-assay variation calculated for 5 samples from the results of 8 replicates was 5.7-10.2% for IgG, 5.8-8.2% for IgM, 5.6-11.3% for IgA, and 7.7-10.9% for IgE. The inter-assay precision was calculated for the same 5 samples assayed in 6 different runs. The inter-assay variations were 7.8-12.7%, 9.5-15.5%, 10.6-15.3%, and 11.1-18.5%, respectively, for IgG, IgM, IgA, and IgE. For testing of specificity of IgE antibodies, 10 different sera, with ODs between 0.32 and 1.98, were examined simultaneously by commercially available mold-specific IgE ELISA assay. Compared with the ELISA described in the Method and Materials section, the overall correlation was 88%.

Absorption of Stachybotrys antibodies with HSA. Stachybotrys mycotoxins bound to HSA were used in inhibition studies and for examination of antibody specificity. Three different sera with high levels of IgG, IgM, or IgA against Stachybotrys, and 3 sera with high levels of antibodies against satratoxin and trichothecene were absorbed with nonspecific and specific antigens. Results showed that nonspecific proteins--such as HAS--did not change the IgG, IgM, and IgA antibody levels against Stachybotrys and mycotoxins; however, Stachybotrys antigens, satratoxin, satratoxin-bound HSA, trichothecene, or trichothecene-bound HSA absorbed the IgG antibody levels from 42-61%, 15-40%, 33-44%, 12-34%, and 24-51%, respectively. Similar to IgG--but to a lesser degree--IgM and IgA antibodies were absorbed more effectively with Stachybotrys antigens than with mycotoxins bound to HSA or with mycotoxins alone. This significant absorption and inhibition of IgG, IgM, and IgA antibodies by fungal antigens of mycotoxins bound to carrier proteins is the best evidence for the specificity of fungal antibodies.

Discussion

Health effects from exposure to molds in water-damaged building environments can result in allergy, infections, or mucous membrane and sensory irritation toxicity alone, or in combination. However, despite this variety of adverse health effects, significant emphasis is placed only on Type I allergy. (29-31)

There are hundreds of molds with thousands of different antigens that can contaminate indoor air. Depending on the types of molds and antigens, as well as an individual's genetic susceptibility, 1 or all 4 types of allergic reactions are possible following exposure. This means that atopic individuals exposed to molds found indoors can develop sensitization and allergy, which can be confirmed by a skin test or RAST IgE test. However, the nonatopic individual, as a result of a lack of IgE production, would not be identified as having mold allergy, even while presenting symptoms of chronic illnesses. (32) For this reason, mechanisms other than IgE mediation have been explored by various investigators. In an epidemiological and immunological study of materials from a water-damaged office building, samples were contaminated with Stachybotrys atra containing mycotoxins. Employees exhibited low levels of abnormalities--in both their cellular and humoral immunities. Although 48

individuals were possibly exposed to Stachybotrys, only 4 had an elevated antibody test for it. (17) Other investigators in several case-control studies have used serology in investigations of subjects from buildings with large amounts of S. chartarum and have found statistically nonsignificant differences in S. chartarum IgG or IgE antibody levels between exposed and control groups. (16-18) Conversely, a recent cross-sectional comparison study of water-damaged or mold-contaminated homes in Finland found fungal-specific IgG concentrations in the sera of residents in houses with and without mold, with other cases showing a tendency for higher antibody levels to most fungi than in control groups. (33)

In another study, following administration of both strains of S. atra and spores, minor changes in hematological parameters were detected in mice ($p < 0.05$); however, IgG antibodies to S. atra could not be detected in the sera of mice exposed to S. atra spores intranasally. (34)

Raunio et al. (20) measured IgE, IgG, and IgA antibodies against S. chartarum in patients with asthmatic or mycotoxicosis symptoms. They reported that there was a better correlation between the IgA response and exposure to the fungus than was found with the IgG response. For the reasons stated earlier, we tested IgG, IgA, IgM, and IgE antibodies against the 7 most common molds found in water-damaged buildings, as well as against 2 different mycotoxins produced by some of these molds, including Stachybotrys.

Data presented in Table 4 showed that at a cutoff point of 2 standard deviations above the mean of controls, a significant percentage of controls had elevated IgG, IgM, and IgA antibodies against mold and mycotoxins. This elevation of mold-specific antibodies in healthy controls is an indication of widespread exposure of the population to indoor molds. At the time of venous blood drawing, these individuals did not present the neurological and behavioral symptoms found in patients exposed to toxigenic molds. Furthermore, presentation of clinical symptomatologies did not correlate with the levels of mold antibodies in all patients. Therefore, it seems that antibody levels do not correlate with disease severity, but are indicative of exposure. Therefore, in some individuals, detected mold IgG, IgM, and IgA antibodies may be protective--but not pathogenic. The question remains: "Why are antibody levels not correlated with disease?" Genetics and epidemiological studies suggest that "You and I can be exposed to exactly the same amount of antigens or haptenic chemicals, but our responses will differ, perhaps by a hundred-fold." (35-37) This variation in the ability to handle antigens and haptenic chemicals depends on genetic susceptibility and enzyme polymorphism. In this regard, numerous specific genetic polymorphisms in multigene families of cytochrome P450 and glutathione s-transferase have been described in humans during the past decades. (36)

In the health-effect survey, investigators reported that in 56% of workers exposed to toxigenic fungi, 33% of the controls had Multiple Chemical Hypersensitivity. (17) In our questionnaire, 72.5% of mold-exposed vs. 25% of controls reported chemical sensitivity (Table 2). Therefore, a high percentage of controls, who had high levels of antibodies against molds and mycotoxins, may not be genetically susceptible to mold antigens and mycotoxins and, hence, do not show the same health-effect responses as do a selective number of workers exposed to toxigenic fungi. In addition, we have no information about the degree or level of exposure to fungal antigens in the control group, or whether individuals with high levels of antibodies will develop symptomatologies in the future. Moreover, the fungal antibodies found in healthy controls could be protective or cross-reactive, inasmuch as some fungal antigens may cross-react with one another, (38) and sometimes they even completely cross-react with different antigens (e.g., Aspergillus and human myelin basic protein (39) or Aspergillus and superoxide dismutase homology (40)). Despite these cross-reactive or nonspecific antibodies in healthy controls, data presented in Figures 1-4 reveal significant differences in the mean [+ or -] standard deviation of controls vs. patients.

Overall, for the majority of tested molds and mycotoxins, the IgA and IgG levels were significantly higher in the patients than in the control group ($p < 0.01$). In patients, the IgM antibody levels against Stachybotrys, Cladosporium, Alternaria, Aspergillus, and mycotoxins--but not against Chaetomium, Epicoccum, and Penicillium--were significantly different from levels in controls (p [less than or equal to] 0.05). With respect to IgE values, whereas the overall mean [+ or -] SD was higher in patients than controls, only antibodies to Aspergillus and satratoxin reached a statistical significance level of p [less than or

equal to] 0.05 (Fig. 4). Similar to the results of earlier studies, (16-18) we found nonsignificant differences in Stachybotrys IgE antibody levels in exposed and control groups (p = 0.09). In our study, the increase in IgE antibody levels against Aspergillus and satratoxin may be explained on the basis of recent studies in mice (41-43) and humans, (44,45) both of which have shown that a T[H.sub.1]/T[H.sub.2] dysregulation and switch to T[H.sub.2] immune response is responsible for hyperimmunoglobulin E syndrome (46)

Whereas our overall antibody test results against mold antigens correlated with the IgG and IgA antibody levels against Stachybotrys reported earlier, (20) differences in antibody levels may stem from antigen preparation and dilution of serum. (17,18) Other investigators cultured the fungus and suspended mycelium in PBS containing PSF, extracted the antigens by homogenization and incubation on the shaker for 16-18 hr at 4[degrees]C, and used human sera at dilutions of 1:10, 1:40, or 1:25, for IgE, IgG, and IgA analysis, respectively. (20) One weakness of the aforementioned studies--ours included--stems from a lack of examination of antibodies against molds not isolated from the building(s) and grown under the same conditions.

We prepared our fungal antigens similar to the method described above, but with some modification. (20) We used similar culture and suspension techniques, but we used repeated sonications during 24 hr of extractions. This resulted in a higher yield of fungal antigens in suspensions. We then compared the protein concentration of commercially prepared fungal antigens with our antigen preparations before and after sonication. The protein concentration of commercial antigens ranged from 0.4 to 2.1 mg/ml, whereas in our preparations, protein concentrations ranged from 0.8 to 3.5 mg/ml prior to sonication. After sonication for 10 times, the concentration of proteins increased to 5.8-16.5 mg/ml. Moreover, by performing SDS gel electrophoresis on an equal amount of protein (i.e., 1 mg [depending on mold species]), we detected between 9 and 22 bands in commercial antigens, between 7 and 16 bands in our preparations before sonications, and between 21 and 36 protein bands after repeated sonications (data not shown). Therefore, we used these antigens with a maximum number of fungal antigens at a concentration of 1[micro]g/well on ELISA plates and found that human sera--dilutions of 1:2 for IgE and 1:100 for IgG, IgM, or IgA in serum diluent--resulted in optimal ELISA optical densities.

The determination of IgG, IgM, and IgA antibody levels, at sera dilutions of 1:100, was based on hundreds of ELISA assays performed in our laboratory. Also, many commercially available ELISA kits use dilutions of either 1:100 or 1:200 for the determination of IgG antibodies in serum. Moreover, in this study, 20 different sera diluted at 1:25, 1:50, 1:100, 1:200, and 1:400 were measured for antibody levels. For all tested sera, the curve at dilutions of 1:50 to 1:200 was linear and, therefore, the IgG, IgM, and IgA antibody detection was performed at 1:100 dilution of serum.

Given the isotype competition from IgG, IgM, and IgA with serum concentrations of 0.5-16 mg/ml, the ideal method for measuring the IgE level is to capture all IgE from a sample in which anti-IgE antibodies are used, or to remove the other immunoglobulins from the serum; for example, absorption of the serum by anti-IgG, IgM, IgA, or with Protein A. (47) However, given that the antibodies used in our ELISA assays are specific and affinity-purified, exhibiting no cross-reactivity to other immunoglobulin classes, we did not perform any absorption studies for IgE assay.

This simultaneous detection of 1 or more classes of antibodies against all molds and mycotoxins suggests cross-reaction between different mold antigens, as has been shown in earlier studies. (38) In addition, simultaneous detection of antibody classes against 2 different mycotoxins (satratoxin and trichothecene) indicates that spores containing mycotoxins can behave as an antigen and, by their presentation to the cells involved in the immune system, result in antibody production against mold antigens--as well as metabolites such as mycotoxins.

To our knowledge, there has been no study concerning the effects of fungal exposure on serum IgG, IgA, IgM, and IgE levels against several molds' antigens, in particular against the mycotoxins. These antibodies appear to be specific, inasmuch as in our absorption studies only specific antigens such as Stachybotrys, or mycotoxins bound to HSA, could reduce the antibody levels against Stachybotrys, satratoxin, or trichothecene by up to 60%, whereas free satratoxin and trichothecene were

significantly less effective in absorbing these antibodies.

Finally, the carrier (HSA) alone did not affect the levels of these antibodies. This fact provides further support for the specificity of these antibodies. Inhibition of these antibodies with mold antigens and mycotoxins, with the simultaneous presence of IgG, IgA, or IgM antibodies against different molds and mycotoxins (Figs. 1-4), lead us to conclude that these specific antibodies could be used only in a subpopulation of patients or in epidemiologic investigations of mold and mycotoxin exposure. In this regard, our finding of a significant percentage of mold-exposed patients with normal levels of antibodies, as well as a few controls with abnormal levels of mold antibodies, warrants further investigation. In future studies, the issues related to antigenic load, duration of exposure, genetic variabilities, and protective vs pathogenic antibodies should be addressed.

Table 1.--Tape Transfers and Microscopic Examination of Bulk Samples and Swab Samples for Viable Microbial Activity on Malt Extract Agar and Cellulose Agar

Sample no.	Results of tape transfer and microscopic mold identification	Percentage
1	Stachybotrys spores	96
	Amorphous dirt and dust	4
2	Stachybotrys spores	98
	Amorphous dirt and dust	2
3	Chaetomium spores and hyphae	60
	Cellulose fiber	20
	Amorphous dirt and dust	20
4	Chaetomium spores	80
	Penicillium and Aspergillus spores	15
	Amorphous dirt and dust	5
5	Alternaria spores	95
	Amorphous dirt and dust	5
6	Chaetomium spores and hyphae	20
	Amorphous dirt and dust	80
7	Stachybotrys spores	0
	Amorphous dirt and dust	100

Sample no.	Swab samples for viable microbial activity: Mold identification	CFU/swab
1	Viable molds	0
2	Stachybotrys sp.	> 2,000
3	Viable molds	0
4	Chaetomium sp.	> 2,000
	Penicillium sp.	> 2,000
5	Epicoccum sp.	> 2,000

	Penicillium sp.	270
6	Epicoccum sp.	560
	Penicillium sp.	> 2,000
7	Viable molds	0

Note: CFU = colony-forming units.

Table 2.--Air Samples for Viable Microbial Activity on Total Bioaerosols

Sample no.	Mold identification	CFU/[m.sup.3]
1	Cladosporium	1,140
	Aspergillus	340
	Penicillium	340
	Myxomycetes, smuts	160
	Others	<100
	Total count	2,200
2	Cladosporium	390
	Ascospores	30
	Hyphae fragments	60
	Myxomycetes, smuts	30
	Total count	510
3	Cladosporium	31,550
	Myxomycetes, smuts	414
	Leptoshaeria	234
	Alternaria	18
	Bipolaris	17
	Ascospores	18
	Total count	32,251
4	Cladosporium	28
	Myxomycetes, smuts	14
	Total count	42

Table 3.--Percentage of Different Symptoms or Abnormal Examinations in Controls (n = 40) and Patients Exposed to Molds (n = 40)

Symptom	Percentage positive in controls	Percentage positive in mold-exposed patients
Fatigue	20.0	92.5
Memory disturbance	10.0	72.5
Depression	10.0	52.5
Anxiety	12.5	52.5
Headaches	22.5	87.5
Blurred vision	0.0	62.5
Lightheadedness	15.0	60.0

Ringing in ears	5.0	62.5
Muscle and joint aches	25.0	75.0
Recurrent flu-like illnesses	5.0	55.0
Severe allergies	17.5	67.5
Chest pain and cough	10.0	70.0
Out of breath easily	15.0	80.0
Multiple Chemical Sensitivity	25.0	72.5
Pulmonary function test	0.0	60.0
SPECT scan	0.0	85.0

Note: SPECT = single-photon emission computed tomography.

Table 4.--Percentage Elevation of Antibodies against Different Molds and Mycotoxins above the Mean ([+ or -] 2 Standard Deviations) of Control Optical Density in Healthy Controls and Patients Exposed to Molds

	Immunoglobulin G		Immunoglobulin A	
Molds and mycotoxins	Control	Patient	Control	Patient
Alternaria	15.0	25.0	12.5	35.0
Aspergillus	12.5	30.0	12.5	22.5
Chaetomium	15.0	42.5	12.5	30.0
Cladosporium	10.0	20.0	10.0	30.0
Epicoccum	12.5	30.0	12.5	22.5
Penicillium	10.0	40.0	10.0	30.0
Stachybotrys	12.5	35.0	17.5	42.5
Satratoxin	12.5	27.5	7.5	22.5
Trichothecene	12.5	25.0	10.0	25.0

	Immunoglobulin M		Immunoglobulin E	
Molds and mycotoxins	Control	Patient	Control	Patient
Alternaria	7.5	15.0	10.0	20.0
Aspergillus	7.5	20.0	10.0	25.0
Chaetomium	10.0	22.5	10.0	25.0
Cladosporium	10.0	25.0	7.5	15.0
Epicoccum	7.5	15.0	10.0	15.0
Penicillium	12.5	20.0	12.5	12.5

Stachybotrys	12.5	40.0	10.0	27.5
Satratoxin	10.0	27.5	7.5	25.0
Trichothecene	5.0	20.0	7.5	20.0

References

(1.) Gravesen S, Nielsen PA, Iverson R, et al. Microfungal contamination of damp buildings--examples of risk constructions and risk materials. Environ Health Perspect 1999; 107(suppl 3):505-08.

(2.) Burge HA. Bioaerosols: prevalence and health effects in the indoor environment. J Allergy Clin Immunol 1990; 86: 687-704.

(3.) Flannigan B, McCabe EM, McGarry F. Allergenic and toxigenic microorganisms in houses. J Appl Bact Symp (suppl) 1991; 70:61S-73S.

(4.) Samson RA. Occurrence of molds in modern living and working environments. Eur J Epidemiol 1985; 1:54-61.

(5.) Baxter CS, Wey HE, Burg WR. A prospective analysis of the potential reaction associated with the inhalation of aflatoxin-contaminated grain dusts. Food Cosmet Toxicol 1981; 19:763-69.

(6.) Croft WA, Jarvia BB, Yatawara CS. Airborne outbreak of trichothecene. Atmos Environ 1986; 20:549-52.

(7.) Jarvis BB, Salemme J, Morais A. Stachybotrys toxins. Nat Toxins 1995; 3:10-16.

(8.) Jarvis BB, Zhou Y, Wang S. Toxigenic molds in water damaged buildings: dechlorogriseofulvins from Memnoniella echinata. J Nat Prod 1996; 59:553-54.

(9.) Williams PP. Effects of T-2 mycotoxin on gastrointestinal tissues: a review of in vivo and in vitro models. Arch Environ Contam Toxicol 1989; 18:374-87.

(10.) Cresia DA, Thurman JD, Jones LJ III, et al. Acute inhalation toxicity of T-mycotoxin in mice. Fundam Appl Toxicol 1987; 8(2):230-35.

(11.) Flannigan B, Miller JD. Health implications of fungi in indoor environments--an overview. In: Samson RA, Flannigan B, Flannigan ME, Verhoeff AP, Adan OCG, Hoekstra ES (Eds). Health Implications of Fungi in Indoor Environments. Air Quality Monographs. Vol 2. Amsterdam: Elsevier, 1994; pp 3-28.

(12.) Gareis M. Cytotoxicity testing of samples originating from problem buildings. In: Johanning E, Yang CS (Eds). Proceedings of the International Conference: Fungi and Bacteria in Indoor Environments: Health Effects, Detection and Remediation. Saratoga Springs, NY, 6-7 Oct, 1995; pp 139-44.

(13.) Jakab GJ, Hmieleski RR, Hemenway DR, et al. Respiratory aflatoxicosis: suppression of pulmonary and systemic host defenses in rats and mice. Toxicol Appl Pharmacol 1994; 125:198-205.

(14.) Nikulin M, Reijula K, Jarvis BB, et al. Experimental lung mycotoxicosis in mice induced by Stachybotrys atra. Int J Exp Pathol 1996; 77:213-18.

(15.) Bush RK, Portnoy JM. The role of abatement of fungal allergens in allergic diseases. J Allergy Clin Immunol 2001; 107:S430-S440.

(16.) Hodgson MJ, Morey P, Leung WY, et al. Building-associated pulmonary disease from exposure to Stachybotrys chartarum and Aspergillus versicolor. J Occup Environ Med 1998; 40(3):241-49.

(17.) Johanning E, Biagini R, Hull D, et al. Health and immunology study following exposure to toxigenic fungi (Stachybotrys chartarum) in a water-damaged office environment. Int Arch Occup Environ Health 1996; 68: 207-18.

(18.) Johanning E, Landsbergis P, Gareis M, et al. Clinical experience and results of a sentinel health investigation related to indoor fungal exposure. Environ Health Perspect 1999; 107(suppl 3):189-94.

(19.) Pestka JJ, Moorman MA, Warner RL. Dysregulation of IgA production and IgA nephropathy induced by trichothecene vomitoxin. Food Chem Toxicol 1989; 27: 361-68.

(20.) Raunio P, Pasanen AL, Husman T, et al. Exposure to Stachybotrys chartarum induces immunoglobulin A antibody response in man. In: Johanning E (Ed). Bioaersols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control. Albany, NY: Eastern New York Occupational and Environmental Health Center, 1999; pp 174-78.

(21.) Knutsen AP, Mueller KR, Hutcheson PS, et al. Serum anti-Aspergillus fumigatus antibody immunoblot and ELISA in cystic fibrosis with allergic bronchopuimonary aspergillosis. J Clin Immunol 1994; 93:926-31.

(22.) Ojanen T, Terho EO, Tukiainen H, et al. Class-specific antibodies during follow-up of patients with farmer's lung. Eur Respir J 1990; 3:257-60.

(23.) Apter AJ., Greenberger PA, Liotta JL, et al. Fluctuation of serum IgA and its subclasses in allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 1989; 84: 367-72.

(24.) Achatz G, Oberkofler H, Lechenauer E, et al. Molecular cloning of major and minor allergens of Alternaria alternata and Cladosporium herbarum. Mol Immunol 1995; 32:213-27.

(25.) Paris S, Fitting E, Ramirez E, et al. Comparison of different extraction methods of Alternaria allergens. J. Allergy Clin Immunol 1990; 85:941-48.

(26.) Portnoy J, Pacheco F, Ballam Y, et al. The effect of time and extraction buffers on residual protein and allergen content of extracts derived from four strains of Alternaria. J Allergy Clin Immunol 1993; 91:930-38.

(27.) Raunio P, Karkkainen M., Virtanen T, et al. Preliminary description of antigenic components of Stachybotrys chartarum. Environ Res 2001; 85:246-55.

(28.) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

(29.) Brostoff J. Immunological mechanisms. In: Brostoff J, Challacombe SJ (Eds). Food Allergy and Intolerance. Eastbourne, U.K.: W.B. Saunders, 1987; pp 433-55.

(30.) Gell PGH, Coombs RRA. Clinical Aspects of Immunology. Oxford, U.K.: Blackwell, 1963.

(31.) Saxon A, Diaz-Sanchez D, Zhang K. The allergic response in host defense. In: Rich RR, Fleisher TA, Schwartz BD, Shearer WT, Strober W (Eds). Clinical Immunology. St. Louis, MO: Mosby, 1995.

(32.) Pope AM, Patterson R, Burge HA (Eds). Indoor Allergens: Assessing and Controlling Adverse Health Effects. Committee on the Health Effects of Indoor Allergens, Division of Health Promotion and Disease Intervention, Institute of Medicine. Washington, D.C.: National Academy Press, 1993.

(33.) Hyvarinen A, Reiman M, Meklin T, et al. Fungal exposure and IgG-levels of occupants in houses with and without mold problems. In: Johanning E (Ed). Bioaersols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control. Albany, NY: Eastern New York Occupational and Environmental Health Center, 1999; pp 166-68.

(34.) Nikulin, M., Reijula, K., Jarvis, B.B., et al. Effects of intranasal exposure to spores of Stachybotyrs atra in mice. Fundam Appl Toxicol 1997; 35:182-88.

(35.) Caldwell M. Beyond the lab rat. Discover 1996; 17: 70-75.

(36.) Perera FP, Hemminki K, Grzybowska E, et al. Molecular and genetic damage from environmental pollution in Poland. Nature 1992; 360:256-58.

(37.) Perera FP. Uncovering new clues to cancer risk. Sci Am 1996; 274:54-62.

(38.) Halsey J. Performance of a Stachybotrys chartarum serology panel [abstract]. Presented at the Western Society of Allergy, Asthma and Immunology Annual Meeting. Allergy Asthma Proc 2000; 21(3):174-75.

(39.) Grogan JL, Kramer A, Nogai A, et al. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. J Immunol 1999; 163:3764-70.

(40.) Flukigers S, Mittl PRE, Scapozza L, et al. Comparison of the crystal structure of human manganese superoxide dismutase and the homologous Aspergillus fumigatus allergen at 2-[Angstrom] resolution. J Immunol 2002; 168:1267-72.

(41.) Cenci E, Perito S, Enssle KH, et al. Th1 and Th2 cytokines in mice with invasive aspergillosis. Infect Immun 1997; 65:564-71.

(42.) Cenci E, Mencacci A, Fe d'Ostiani C, et al. Cytokine and T helper-dependent lung mucocal immunity in mice with invasive pulmonary aspergillosis. J Infect Dis 1998; 178: 1750-64.

(43.) Cenci E, Mencacci A, Del Sero G, et al. IL-4 causes susceptibility to invasive pulmonary aspergillosis through suppression of protective type 1 responses. J Infect Dis 1999; 180:1957-62.

(44.) Roilides E., Sein T, Roden M, et al. Elevated serum concentrations of interleukin-10 in non-neutropenic patients with invasive aspergillosis. J Infect Dis 2001; 183:518-25.

(45.) Wolach B, Alon E, Gottesman G, et al. Pulmonary aspergillosis in a child with hyperimmunoglobulin E syndrome. J Infect Dis 1998; 26:204-09.

(46.) Bozza S, Gaziano R, Spreca A, et al. Dendridic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate TH responses to the fungus. J Immunol 2002; 168:1362-71.

(47.) Wojdani A, Etessami S, Cheung G. IgG is not the only inhibitor of IgE in the RAST test. Ann Allergy 1985; 55: 463-68.

Submitted for publication March 18, 2003; revised; accepted for publication October 4, 2003.

Requests for reprints should be sent to Dr. Aristo Vojdani, 8693 Wilshire Blvd., Suite 200, Beverly Hills, CA 90211.

E-mail: immunsci@ix.netcom.com

ARISTO VOJDANI

Immunosciences Laboratory, Inc.

Section of Immunology

Beverly Hills, California

ANDREW W. CAMPBELL

Medical Center for Immune and Toxic Disorders

Spring, Texas

ALBERT KASHANIAN

ELROY VOJDANI

Immunosciences Laboratory, Inc.

Section of Immunology

Beverly Hills, California

Disconnection of man and the soil: Reason for the asthma and atopy epidemic?

Leena von Hertzen, PhD, and Tari Haahtela, MD, PhD *Helsinki, Finland*

Intense search has been going on to find factors responsible for the asthma and atopy epidemic in Western societies. Attention has increasingly been devoted to environmental saprophytes, which, in addition to gut commensals, might be the major players in the development and fine tuning of immunologic homeostasis. This review outlines current evidence for the role of environmental saprophytes in the development of atopic disease and considers the consequences of urbanization in reducing contacts with soil microorganisms. The major microbial components that have been shown to possess immunomodulatory capacity and their respective Toll-like receptors are also discussed, as are the possible mechanisms underlying the ability of saprophytes to confer protection against atopic disease. (J Allergy Clin Immunol 2006;117: 334-44.)

Key words: *Allergy, asthma, atopy, hygiene hypothesis, saprophytes, urbanization*

The current asthma and atopy epidemic in Western societies has raised a common concern and questions of factors involved. Although in some countries prevalence rates in atopic diseases appear to have leveled off,[1-4] trends are still on the increase in many other countries.[5,6]

Numerous studies have consistently shown that high asthma and atopy rates are associated with urbanization and Western lifestyle.[7,8] Accumulating data suggest that something that is necessary for the normal maturation of the immune system might be lacking in our affluence.[9] Conversely, farm environment and a more traditional lifestyle in nonaffluent countries appear to confer protection against atopic disease.[8,10,11] Although the ultimate factors responsible for the asthma and atopy epidemic have remained unidentified, a common denominator for both living on a farm and in a nonaffluent environment is the heavy exposure to microorganisms in soil and vegetation.

Most of the microorganisms we encounter do not cause any overt infection but are still recognized by the innate immune system. Microbes in this respect need not be alive because even nonviable microbial components interact with the innate immune system. Persistent and moderate environmental exposure to microbial components might play a decisive role in the normal maturation of the immune system in childhood.[12] It has been proposed that certain microorganisms that have been present throughout the mammalian evolutionary history are recognized by the innate immune system as ''no danger'' signals and thus do not trigger inflammatory responses but instead have the ability to induce tolerance through rapid regulatory T (Treg) cell responses.[13] These organisms include saprophytic mycobacteria, lactobacilli, and some intestinal parasites that are able to elicit Treg cell responses *in vivo*[14-17] and *in vitro*.[18] The list of such microbes will certainly grow in the next few years.

The focus of the research in the context of the hygiene hypothesis has largely shifted from overt infections and the T_H1/T_H2 paradigm to noninfectious organisms, Treg cells, and Toll-like receptors (TLRs), as new data have been accumulated and the paradigm was found to be unable to unambiguously explain some important epidemiologic findings.[19,20] Indeed, diseases of immune dysregulation, including atopic diseases, are now considered to develop, more or less, as a result of failure in Treg cell function.[13] Immune defense mechanisms that evolved during the long history of humankind in a hostile environment appear now to be less appropriate when living in a clean environment.[21]

Abbreviations used
TLR: Toll-like receptor
Treg cell: Regulatory T cell

HYPOTHESIS

In this review we propose a hypothesis that one major factor in the current asthma and atopy epidemic might be the disconnection of man and the soil.

EVIDENCE TO SUPPORT THE HYPOTHESIS

There is abundant literature on adverse respiratory health effects attributable to exposure to environmental

From the Helsinki University Central Hospital, Skin and Allergy Hospital.
Supported by the Academy of Finland (grant no. 201246), by Helsinki University Hospital Grants (no. 2250 and 5201), and by the Finnish Anti-Tuberculosis Association Foundation.
Received for publication October 7, 2005; revised November 3, 2005; accepted for publication November 10, 2005.
Reprint requests: Leena von Hertzen, PhD, HUCH, Skin and Allergy Hospital, PO Box 160, 00029 HUS, Finland. E-mail: leena.vonhertzen@kolumbus.fi.
0091-6749/$32.00

doi:10.1016/j.jaci.2005.11.013

bioparticles (eg, bacteria, molds, and fugal spores).[22] Paradoxically, data are now accumulating to suggest that exposure to microbes in soil and vegetation might be beneficial, even necessary, for the normal maturation of the immune system.

Several lines of evidence indicate that settings associated with high-level exposure to microorganisms in soil are associated with reduced risk for asthma and atopy. Such settings include farm environments, environments in nonaffluent Eastern countries, and rural areas, particularly in developing countries.

Farm environment and atopic disease

More than 30 studies from the last 6 years have consistently shown that children who have lived or are living on a farm are less likely to have atopic disease than their counterparts not living on a farm. The issue of farming and atopic disease has been thoroughly reviewed elsewhere and is not reiterated here.[8,10,23] In many of these studies, the effect of parental farming on the development of atopic disease in the child has been found to be dose dependent,[24-26] and many of these studies have also revealed ''frequent contacts with farm animals'' as one of the major factors responsible for this effect.[25,27,28] However, frequent contacts with farm animals can also be a surrogate marker for exposure to microorganisms in soil and vegetation because farm animals (and pets) are likely to serve as a secondary source of exposure to such microorganisms. In addition, frequent contacts with farm animals could also reflect general activity of the child on the farm. The effect of farming on conferring protection against asthma and atopy might not be restricted to early life only because current parental farming has been found to be an even stronger protective factor than that in early life.[28]

Environments in nonaffluent societies: The effect of traditional lifestyle

Frequent compost and waste handling, wood handling, and animal excreta and manure handling are examples of high-level microbial exposure[22] associated with a traditional lifestyle. Unchlorinated surface water from lakes and rivers might be used as domestic water, untreated waste water might be used for irrigation, and animal excreta might be used as manure. Traditional lifestyle might also be associated with a microbe-rich diet (eg, frequent use of fermented vegetables).[20] We found recently that occurrences of atopy (determined by means of skin prick tests) and atopic diseases were substantially lower among schoolchildren and their mothers in Russian Karelia compared with that seen in their counterparts in North Karelia, Finland, irrespective of the geographic proximity of the areas and similar geoclimatic and vegetative conditions (see Fig E1 in the Online Repository at www.jacionline.org). Analysis of generational differences revealed that in Finland children had higher atopy rates than their mothers, whereas in Russia the opposite trend, children having lower atopy rates than their mothers, emerged. No signs of westernization, with atopy prevalence as a proxy, were yet discernible in Russian Karelia, which was part of the Soviet Union until 1991.[29] The results are in line with those reported earlier from other Eastern countries in transition crisis.[11] The East-West gradient in light of the occurrence of atopic diseases has been thoroughly reviewed.[8]

Rural areas and atopic disease: Evidence from relocation studies

Data both from Western and particularly from developing countries, in which great differences in lifestyle still exist between urban and rural areas, have shown that living in rural areas might confer protection against atopic disease, even in a dose-dependent manner.[30-34] A recent study in Mongolia that compared the occurrence of atopy and allergic disorders in 3 different environments of various degree of urbanization—a city area, rural towns, and villages—found significant increasing trends in the prevalence of allergic rhinitis and atopy, as determined by using skin prick tests, with increasing degree of urbanization.[30] Analysis of the effect of relocation revealed that continuous living in a village since birth was most protective against atopy and allergic rhinitis, whereas those who relocated from villages to towns in adolescence or adulthood acquired allergic conditions at rates approaching those found in subjects who had always lived in towns.[35] The results are in line with those of other migrant studies showing that sensitizaton rates and profiles among immigrants shift along with time, resembling finally those in natives,[36,37] thus supporting the view that there might not be any strictly limited window period in early life during which the individual is susceptible to immunomodulatory effects of the environment; rather, susceptibility to immunomodulation probably continues to adolescence, even to adulthood.[38-40] However, it must be borne in mind that disparities between asthma and atopic conditions in this respect might exist.[10]

Indicators of urbanization and atopic disease

Before urbanization, humans have lived in close contact with soil, either directly or indirectly through food, water, and air,[41] and heavy exposure to environmental microorganisms has occurred through inhalation, ingestion, and skin contact.[20] Inhalation of bioaerosols (composed of microbes and their components, such as products of plants and fecal material from animals) has been considered to represent the major route of exposure.[22] This natural exposure to microbes, particularly in soil, has been dramatically reduced along with urbanization characterized by living in environments covered with asphalt and concrete.

There are no unambiguous and commonly accepted criteria for urbanization. Many of the suggested criteria are based on population density and are not relevant for sparsely populated countries, such as Finland.

We performed time-series analyses of occurrence of atopic diseases and urbanization using the asphalt index (use of asphalt, tons per inhabitant per year, years 1960-1990; The Road Administration, the Ministry of Traffic and Communication, and The Finnish Asphalt

Association. Census statistics; Statistics Finland; http://statfin.stat.fi) and the decreasing proportion of farmers among the population (years 1966-2000, Statistics Finland; http://statfin.stat.fi) as indicators of urbanization here because both are closely related to reduced contacts with soil.

Prevalences of asthma and allergic rhinitis were based on our recent data on occurrence of atopic disease among military conscripts.[6] The database here covered the years 1966 through 2000 and comprised more than 1 million military conscripts aged 18 to 19 years. The men had been examined to establish their fitness for service at the call up. Similar diagnostic codes for asthma and allergic rhinitis have been used throughout the study period on the basis of ICD-8 and ICD-9 in 1966 through 1996 and ICD-10 in 1997 through 2000.

We found that the use of asphalt, which in Finland started at the end of the 1950s and was very modest still in the early 1960s (The Road Administration and The Finnish Asphalt Association, unpublished data), increased 10-fold in 3 decades. A nearly similar increase was also found in asthma prevalence among military conscripts, from 0.3% in 1966 to 2.6% in 1995 during a 30-year period, and the trend was upward for the whole study period (Fig 1, *A*).[6]

Along with urbanization, heavy structural changes have occurred in agriculture and forestry. In Finland, the proportion of farmers among the population has decreased from 17.3% in 1970 to 4.9% in 2000. During the same time, the occurrence of allergic rhinitis, as assessed among young Finnish men, increased almost exponentially, from 0.1% in 1966 to 8.9% in 2000 (Fig 1, *B*).[6] The proportion of population that is continuously in natural connection with soil has thus diminished since the 1960s and will evidently still diminish, whereas the opposite has occurred for the prevalence of allergic rhinitis.

Urbanization can also be characterized by living in apartment houses, which is, similarly to the use of asphalt and decrease in farming occupation, likely to reduce contact with soil. Dwelling type has indeed been shown to affect the magnitude of exposure to microorganisms in the environment. A study among 81 randomly selected teachers showed that both personal exposure to microorganisms (assessed with transportable inhalable aerosol samplers) and microbial concentrations in their homes were higher among persons living in family (single) houses compared with those in apartment houses, and this was considered partly to be due to increased outdoor activities among those living in family houses.[42] A sedentary lifestyle with little outdoor activity might not only be involved in the association between asthma and obesity[43] but can also increase the risk of atopy through reduced exposure to saprophytes in the environment.

Although exposure to pathogens has been found to be inversely associated with atopic diseases[44] and undoubtedly is able to exert immunomodulatory effects in early life, infectious agents might represent only a minimal part of our total exposure to microorganisms. The largely neglected group of saprophytes in the environment might play a decisive role, in addition to gut microbiota,[45] in the development and maintenance of immunologic homeostasis.

Source of drinking water and atopic disease

An important issue closely related to soil is the runoff of soil microorganisms into natural waters[46] and the use of such waters as drinking water. We found that in Russian Karelia, where atopy and atopic diseases are uncommon,[29] surface water bodies, lakes and rivers, are used as domestic water, frequently without any chemical or other treatment. Previous data have shown that consumption of unpasteurized milk in early life is associated with reduced risk of asthma and atopy in later life independently from other determinants.[26] It is reasonable to assume that consumption of untreated surface water could have similar effects and could be involved in the low atopy prevalence in Russian Karelia. Indeed, this view is supported by recent data from Ethiopia showing that consumption of river water in rural areas, as contrasted with consumption of pipe water in urban areas, conferred protection against atopic eczema.[47] In another study among schoolchildren in a rural area of Latin America, consumption of river water was found to be weakly protective against atopy.[48]

SOIL MICROBIOTA

Soil is considered the most complicated biomaterial and at the same time the most diverse and important ecosystem on the planet.[49] The definition of the microbial composition of a typical soil has proved to be problematic because of this diversity of soil types and the complexity and variability of the physicochemical circumstances. Nonetheless, the majority of soil bacteria are considered to belong to the lineage of gram-positive bacteria,[50] and members of the phylum Actinobacter have been found to predominate in the soil.[51] This phylum includes genera such as *Mycobacterium* species, *Streptomyces* species, *Actinomyces* species, *Corynebacterium* species, and *Bifidobacterium* species.[52] Fungi are often dominant in soils in terms of their biomass, particularly fungi dominate in acid temperate or polar soils that are oligotrophic,[53,54] whereas bacteria predominate in near-neutral or moderately alkaline soils.[54] Some estimates of the density of microorganisms in a normal near-neutral organic soil obtained by means of cultivation and microscopy have been reported: Actinobacter, for example, might occur at the concentrations of $10^{9\text{-}13}$ bacteria/dm3 soil and other bacteria at the level of 10^{8}/dm3 soil.[51] Cultivation, which has traditionally been used to measure bacterial densities in soil samples, probably greatly underestimates the true values. The more modern methods, such as PCR tests and fatty acid analyses, have revealed that a considerable proportion of all bacteria in soil is in a dormant (metabolically inactive) stage.

Mycobacteria, one of the major bacterial groups in soil and natural waters, including more than 80 saprophytic species,[13] has received considerable attention during the last decade as a potential immunomodulatory agent in

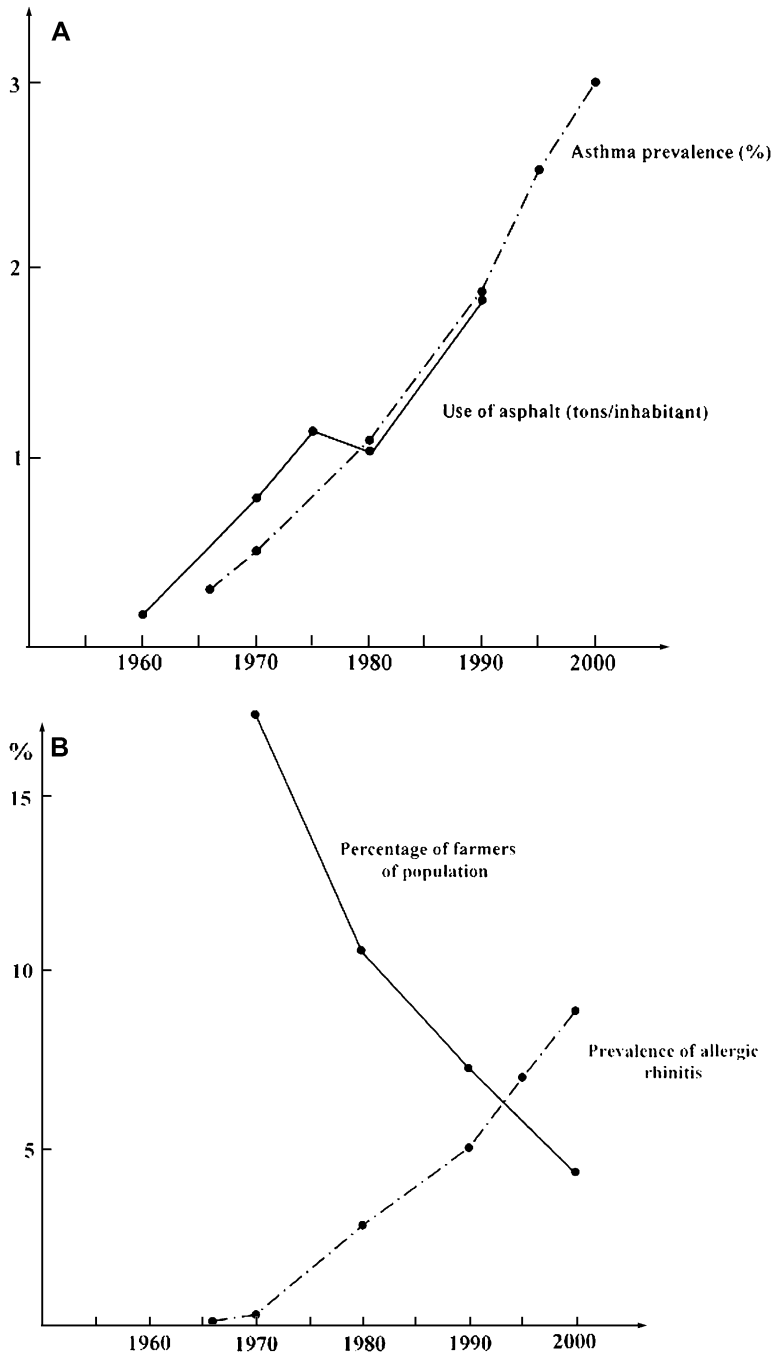


FIG 1. A, Use of asphalt (tons per inhabitant per year) from 1960 through 1990 and occurrence of diagnosed asthma among military conscripts from 1966 through 2000 in Finland.[6] **B**, Proportion of farmers among the whole population from 1970 through 2000 and occurrence of diagnosed allergic rhinitis among military conscripts from 1966 through 2000 in Finland.[6] Supplemental information is available in the Online Repository at www.jacionline.org.

alleviating symptoms of atopic disease[55] and even conferring protection against them.[14,15] In addition, much of the current research of atopic diseases has been focused on lactobacilli, which are considered one of the potential groups of immunomodulatory agents with prophylactic and therapeutic potential.[56-58] Notably, lactobacilli are originally common inhabitants of plants and grow at the expense of the nutrients liberated from decomposing plant material.[59]

Results from our laboratory indicate that, indeed, gram-positive bacteria represent the great majority (90%) of all bacteria in settled barn dust measured by means of

exact fatty acid analyses (Saris et al, unpublished data). However, the proportions of gram-negative and gram-positive bacteria might vary to some extent according to the season,[60] particularly in northern boreal latitudes, where the soil is frozen and covered with snow for months.

MAJOR COMPONENTS OF MICROORGANISMS WITH IMMUNOMODULATORY POTENTIAL AND THEIR INTERACTION WITH THE INNATE IMMUNITY

The idea that microbial products have immunomodulatory potential and could be used as immunotherapeutic agents in asthma and allergies dates back to the 1950s.[61] Bacterial extracts obtained mainly from species associated with upper respiratory tract and urinary tract infections administered subcutaneously were earlier used for such purposes but are not in use today because several double-blind studies showed no efficacy in asthma, possibly because of overly low concentrations of bacterial material in these extracts, nonoptimal route of administration, and overly long intervals between the doses (1 week or longer). No studies on the preventive effect of oral bacterial extracts are available.[62]

Because both viable and nonviable bacterial components have been found to be immunobiologically active, a renewed interest in bacterial cell-wall components has been raised, although the literature on immunologic effects of cell-wall components other than LPS (endotoxin) is relatively scarce. However, in addition to LPS, 2 other ubiquitous bacterial components, lipoteichoic acids and peptidoglycans, might be of importance in this respect, and the cell-wall components of fungi (eg, β-glucans) have also been found to have immunomodulatory potential. In addition, a potent immunomodulator appears to be bacterial DNA, the unmethylated CpG oligonucleotide.

TLRs

TLRs, the receptors that recognize conserved microbial structures, represent an ancient system of host defense but were not discovered until 1989.[63,64] This discovery brought the formerly underappreciated innate immunity into the focus of research. TLRs have been extensively reviewed during the last few years.[64-67] To date, at least 10 different TLRs have been identified in mammals, and they have been found to play a decisive role in recognizing microbes and bridging the innate and acquired immune responses.[66] Although the role of TLRs has been mostly shown in infectious systems, there are good reasons to believe that TLRs are equally important in repeated exposure to saprophytic bacteria in the environment and commensals in the gut and are involved in the induction of tolerance. TLR2, TLR4, and TLR9 are briefly considered here in the light of exposure to environmental saprophytes. TLR2 is the principal receptor and signaling molecule for gram-positive bacteria, lipoteichoic acid, mycobacteria, mycobacterial lipoarabinomannan, bacterial lipoproteins, and fungal β-glucan. TLR4 is the primary receptor for LPS from gram-negative bacteria, and TLR9 recognizes bacterial unmethylated CpG oligonucleotides.[64]

Major cell-wall components of bacteria and fungi

LPS (endotoxin). The chemical composition of LPS, the major cell-wall component of gram-negative bacteria, has been known for more than 50 years, and its physicochemical properties, stability and heat resistance, are also well established.[68] Abundant literature exists concerning the biologic effects of this macromolecule. In several studies the relative lack of exposure to endotoxin has been suggested to be one major reason for the asthma and atopy epidemic.[33,69,70] The relationship between LPS and the occurrence of asthma and atopy has also been thoroughly reviewed elsewhere.[68,71,72]

It has also been known since the 1950s that the biologic activity of LPS resides in the lipid A moiety (the theory of the ''endotoxic principle''[73]). Moreover, it is now well established that the immune response to LPS is dependent on the chemical structure and molecular conformation of the lipid A moiety. Lipid A consists of a phosphorylated glucosamine disaccharide (the backbone), to which fatty acids are attached. It has been found that biologic effects of LPS from different gram-negative bacteria are not similar.[74] The critical determinants here are the length and number of acyl (fatty acid) chains, the asymmetry of these chains, and the number and distribution of negative charges.[74,75] Interestingly, the widely used test to detect LPS in various samples, the Limulus amebocyte lysate test, has been found to be specific for the LPS glucosamine backbone and is thus not a measure of biologic activity. Fatty acid analyses might better correlate with biologic activity because the conformation and number of acyl (fatty acid) chains in lipid A appear to be central to the capacity of LPS to interact with TLRs and to induce cytokine production.[76]

Peptidoglycan and teichoic acid. With the exception of a few groups of some minor bacteria, such as *Mycoplasma* and *Chlamydia* species, all members in the domain Bacteria have one common denominator, the presence of peptidoglycan as the main strengthening and shape-determining constituent of the cell wall. In gram-positive bacteria, peptidoglycan accounts for at least 50%, and often more, of the total dry weight of the wall, but in gram-negative bacteria, it might comprise less than 10% of the dry weight of the wall.[59] In gram-negative bacteria peptidoglycan is localized in the innermost layer of the wall and, although extremely thin, is still capable of retaining the shape of the cell.[59] Peptidoglycan is composed of 2 amino sugars, N-acetylglucosamine and N-acetylmuramic acid, and a side chain of 4 amino acids that can vary from species to species.[77] The other of these amino sugars, N-acetylmuramic acid, is a molecule specific to the domain Bacteria and can thus be used for laboratory diagnostic purposes. In a recent study, van Strien et al[78] showed that muramic acid can be found in dust from children's mattresses and in higher concentrations in those of farmer's children.

Wheezing and asthma were inversely associated with the muramic acid concentration, independently from LPS, whereas no association was found for atopic sensitization. Increased muramic acid concentrations were found in homes heated with wood or coal, independent of whether it was a farm home, which suggests that settings associated with traditional lifestyle and increased exposure to microorganisms, rather than farming per se, is responsible for this effect.

The other characteristic cell-wall components of most gram-positive bacteria are teichoic acids (up to 50% of the dry weight of the wall). Teichoic acids are water-soluble polymers containing ribitol or glycerol residues joined through phosphodiester linkages.[77] In lipoteichoic acids there is a single lipid side chain anchored to the ribitol or glycerol backbone.[79] Teichoic and lipoteichoic acids have been found to exert potent inflammatory responses.[80-82] As stated above, teichoic acids are recognized by TLR2. Peptidoglycan has similarly been considered to be recognized by TLR2,[64,67] but this view has recently been challenged by the identification of intracellular proteins, the nucleotide-binding oligomerization domain 1 and 2, as the principal receptors for peptidoglycan fragments.[83,84]

Fungal cell-wall components. Fungal cell walls differ from those of bacteria by lacking peptidoglycan, teichoic acids, and LPS. In their place are the external and antigenic peptidomannans embedded in matrices of α- and β-glucans, and structural rigidity is provided by chitin.[85] The principal sterol in fungal cell membranes is ergosterol (corresponding cholesterol in mammalian cells), which has been used, in addition to β-glucans, in laboratory diagnostics of environmental samples. The immunobiology of β-glucans has been reviewed earlier.[86] Most of the recent studies on fungal recognition by TLRs have been centered on a few potentially pathogenic fungi, such as *Candida albicans* and *Aspergillus fumigatus*. It has been found that both TLR2 and TLR4 can be important for their recognition[87] and that TLR2 might be involved in maintaining prolonged candidasis by mediating anti-inflammatory signals leading to IL-10 production and generation of regulatory T cells.[88] In addition, a new coreceptor for β-glucan, dectin-1, expressed on macrophages, dendritic cells, and monocytes, has been found to be involved in mediating proinflammatory responses to fungi together with TLR2.[89,90] The recognition of fungi by the innate immune system appears to be more complex than that of bacteria because fungi can exist in 2 forms, as hyphae or conidiae.

Bacterial CpG oligonucleotides

Since the late 1980s, bacterial DNA has been known to possess immunostimulatory properties[91] that are not found in vertebrate DNA.[92] The activating element was identified as an unmethylated CpG oligonucleotide that was found to be 20-fold more abundant in bacterial than in vertebrate DNA, and when present in vertebrate DNA, about 70% of it was found to be methylated.[93]

Unmethylated CpG oligonucleotides have the ability to elicit a multifaceted innate immune response characterized by the production of IL-12, IL-18, and IFN-γ and the upregulation of costimulatory molecules by antigen-presenting cells, B cells, and natural killer cells.[94] They have both direct and indirect effects on the commitment of $CD4^+$ cells to a T_H1 phenotype and are thus able to downregulate or reverse T_H2 responses.[94-96] Mammalian DNA or methylated bacterial DNA, in contrast, does not induce these responses.[97] In addition, CpG oligonucleotides have been found to strongly induce IL-10 release, which inhibits both T_H1 and T_H2 responses in a dose-dependent manner.[98] This IL-10 is the key cytokine in the development of adaptive regulatory T (Treg) cells, which in turn are able to downregulate antigen-specific IgE responses and promote tolerance to allergens.[99,100] Data on synthetic CpG oligonucleotides in murine models of atopic diseases and as vaccine adjuvants and therapeutic agents in human subjects with allergic disorders are promising.[94-96,101]

In a study by Roy et al,[102] bacterial DNA and LPS contents in dust from urban, rural, and farm homes and from farm barns were quantified (by means of PCR specific for bacterial ribosomal DNA and the Limulus test, respectively) to determine whether there are differences in the immune stimulatory capacity between different dust samples. The highest bacterial DNA levels were found in farm barns, followed by rural homes, farm homes, and urban homes. Farm barn DNA significantly potentiated LPS-induced IL-10 and IL-12p40 release from PBMCs, whereas DNA from urban homes did not show this effect, probably because of the low content of bacterial DNA in urban home dust; only approximately 3% of the total DNA content in urban samples was bacterial in origin. Increased IL-10 and IL-12 release shown after stimulation of PBMCs with barn dust DNA and LPS might be crucial in the context of environments conferring protection against atopic diseases.[102] Furthermore, we found a 3.5-fold higher bacterial DNA content (measured by means of bacterial ribosome-specific PCR) in barn dust compared with urban-suburban home dust (5127 vs 1479.5 ng bacterial DNA/g dust; Saris et al, unpublished data). It is known that vertebrate DNA does not possess immunostimulatory capacity but might neutralize or even inhibit the immunostimulatory effects of bacterial CpG motifs.[103,104] The only known TLR for unmethylated CpG oligonucleotides is TLR9.

The innate immunity recognizes saprophytic bacteria, which results in release of proinflammatory cytokines. We have shown *in vitro* that robust responses are elicited in murine macrophages when they are stimulated by common soil microorganisms isolated from barn dust, such as *Streptomyces* species, *Sphingomonas* species, and *Macrococcus* species (Pylkkänen et al, unpublished data), and a dose-dependent and rather similar response was found in the production of, for example, TNF-α for all 3 organisms, contrary to *Bacillus* species, which showed minimal response in this *in vitro* setting (Pylkkänen et al, unpublished data). None of these genera represents true gram-negative bacteria, because *Sphingomonas* species, although categorized as gram-negative, do not possess

LPS but have sphingolipids instead. Interestingly, we found that the dominant (>85%) bacterial genus in dust from urban-suburban homes was *Bacillus* species (Saris and Andersson, unpublished data), lending further support to the view that urban home dust might have minimal, if any, immunomodulatory capacity. Nonetheless, the ability of the common soil saprophytes *Streptomyces* species, *Sphingomonas* species, and *Macrococcus* species to elicit robust proinflammatory cytokine responses *in vitro* raises the question of tolerance, which must have evolved during the long history of coexistence of these saprophytes and man.[13]

TOLERANCE AND Treg CELLS

Repeated or persistent exposure appears to be one fundamental factor in the induction of tolerance. Repeated intranasal antigen exposure leads to decreased bronchial reactivity and tolerance in T_H2-sensitized mice.[105,106] In addition, allergen desensitization therapy (injection of a specific allergen extract at increasing doses) has for years been successfully used, particularly in patients with hay fever and insect venom allergy.[107] Tolerance is mediated by several mechanisms, including anergy and deletion of effector T-cell clones, and particularly by the induction and function of Treg cells.[108] These Treg cells are defined as cells that actively control the function of other cells, mostly in an inhibitory way.[109] Two major lineages of Treg cells have been identified: (1) naturally occurring, thymus-derived Treg cells expressing the transcription factor Foxp3, which are associated primarily with the control of autoantigens, and (2) induced (adaptive), antigen-specific Treg cells, which require IL-10, TGF-β, or both for their differentiation and function.[107,110] These cells ameliorate inflammation through the release of IL-10, TGF-β, or both in repeated or persistent exposure to prevent immune pathology (a form of tolerance) and maintain the persistence of low numbers of antigens in the body, which is necessary in certain cases to provide long-lasting immunity against reinfections.[111] Induction of antigen-specific Treg cells has been performed by administration of heat-killed *Mycobacterium vaccae* and allergen (ovalbumin) into mice. These specific Treg cells were found to release IL-10 and TGF-β and suppress eosinophilia and bronchial hyperresponsiveness.[112] Desensitization therapy has also been found to operate through IL-10.[107] In general, Treg cells are able to prevent the development of highly polarized T_H cells,[113] and one of the mechanisms involved in the development of asthma and atopy has been suggested to be a failure in Treg cell function.[107,109,113] Several excellent review articles of Treg cells are available for further reading.[107,109,110,113]

Treg cells can be preferentially induced at mucosal surfaces, particularly in the gut and respiratory tract.[111] In urbanized Western societies the natural environment might no longer have the ability to maintain the respiratory and gut mucosal system in a state that favors the development of Treg cells and mucosal tolerance to harmless bioparticles.[109] Persistent exposure to saprophytic bacteria in soil and vegetation, in addition to commensals in the gut and respiratory tract, might be needed to stimulate the production of IL-10 and TGF-β through the innate immune system, which in turn are required for the development of inducible Treg cells.

TLRs AND GENE-ENVIRONMENT INTERACTION

Innate immunity is now recognized as a central element also in the gene-environment interaction. The significance of particularly the TLR2 gene in this respect has been demonstrated in several studies. In European children TLR2 gene expression has been found to be higher in blood cells obtained from farmers' children compared with that seen in children not growing up on a farm. A similar difference was not found for the TLR4 gene.[114] Furthermore, a genetic variation in the TLR2 gene was shown to be a major determinant of reduced susceptibility to asthma and atopy in farmers' children but not in nonfarmers' children.[115] No clear association could be found between variations in the TLR4 gene and asthma or hay fever either in farmers' or nonfarmers children in most,[115,116] albeit not all,[117] studies.

These studies provide convincing evidence for gene-environment interactions: a certain polymorphism is expressed only in a certain environment. They also underscore the significance of TLR2 in environments associated with high exposure to soil microorganisms. Because TLR2 is the main receptor for gram-positive bacteria and their structural molecules, lipoteichoic acid and lipoproteins,[64,67] these studies might also point to the significance of particularly gram-positive bacteria in this context. Interaction of TLR2 with its ligands has been found to lead to rapid release of IL-10, which can block the induction of IL-12p35 and IFN-γ by TLR3 and TLR4.[118] It remains to be clarified whether TLR2 is the crucial Toll receptor in mediating IL-10 release for the development of inducible Treg cells.

CONCLUDING REMARKS

Several lines of evidence support the view that the environment in modern industrialized societies is unable to provide the stimulation for the developing immune system that might be beneficial or even necessary: disruption of the ancient connection of humankind and the soil might have had unexpected consequences.

The immunomodulatory role of saprophytic bacteria in soil and vegetation is now increasingly recognized. The innate immune system recognizes such saprophytes or their nonviable components encountered at respiratory and gut mucosal surfaces; however, robust inflammatory responses are not normally elicited but kept in tight control through mechanisms that involve the function of Treg cells that in turn might control the development of atopic

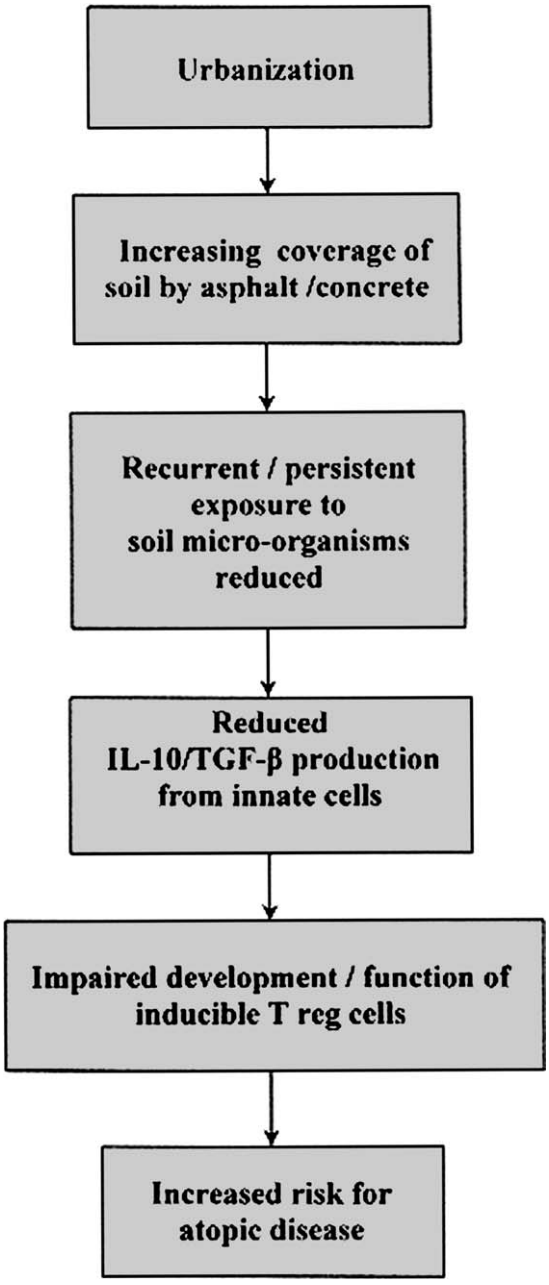


FIG 2. A model of the asphalt theory in the development of atopic diseases. Supplemental information is available in the Online Repository at www.jacionline.org. Because of the implementation of new technologies, including recycling of asphalt material, the figures for the use of asphalt from 1991 onward are not comparable with the earlier ones and have not been included in the analysis. Figures for the years 1960 through 1974 are based on unofficial data from the Road Administration, Ministry of Traffic and Communication, because no official statistics are available for that period. Data on the prevalence of diagnosed asthma and allergic rhinitis are based on Latvala et al.[6]

diseases. A number of microorganisms and their components have been found to induce the production of IL-10, the differentiation factor of inducible Treg cells, by innate cells.[111,119]

Here we proposed a hypothesis that the disconnection of man and the soil might be one major factor in the current asthma and atopy epidemic, as shown in Fig 2. The hypothesis could be tested in animal models and in further comparative and more clearly defined epidemiologic settings, and if correct, a strategy that involves enhancement of the development and activity of Treg cells[117] without concomitant induction of inflammation by bacterial products is evidently the goal to pursue. Central to the outcome of this strategy is probably timing (with respect to the primary sensitization), duration, dose, and route of exposure. The nature of the microbe might also play a role, although it is likely that there is no single agent or agent group behind the protective effect; rather, a mixture, including members of the phylum Actinobacter, might be involved. In addition, the product might not be based on bacterial cell-wall components only because bacterial DNA might have beneficial effects in this respect.

REFERENCES

1. Braun-Fahrländer C, Gassner M, Grize L, Takken-Sahli K, Neu U, Sticker T, et al. No further increase in asthma, hay fever and atopic sensitisaton in adolescents living in Switzerland. Eur Respir J 2004;23: 407-13.
2. Ronchetti R, Villa MP, Barreto M, Rota R, Pagani J, Martella S, et al. Is the increase in childhood asthma coming to an end? Findings from three surveys of schoolchildren in Rome, Italy. Eur Respir J 2001;17:881-6.
3. Verlato G, Corsico A, Villani S, Cerveri I, Migliore E, Accordini S, et al. Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study. J Allergy Clin Immunol 2003;111: 1232-8.
4. Yura A, Shimizu T. Trends in the prevalence of atopic dermatitis in school children: longitudinal study in Osaka Prefecture, Japan, from 1985 to 1997. Br J Dermatol 2001;145:966-73.
5. Bråbäck L, Hjern A, Rasmussen F. Trends in asthma, allergic rhinitis and eczema among Swedish conscripts from farming and non-farming environments. A nationwide study over three decades. Clin Exp Allergy 2004;34:38-43.
6. Latvala J, von Hertzen L, Lindholm H, Haahtela T. Trends in prevalence of asthma and allergy in Finnish young men: a nationwide study from 1966 to 2003. BMJ 2005;330:1186-7.
7. von Mutius E. The environmental predictors of allergic disease. J Allergy Clin Immunol 2000;105:9-19.
8. von Hertzen L, Haahtela T. Asthma and atopy—the price of affluence? Allergy 2004;59:124-37.
9. Ring J, Krämer U, Schäfer T, Behrendt H. Why are allergies increasing? Curr Opin Immunol 2001;13:701-8.
10. von Mutius E. Influences in allergy: epidemiology and the environment. J Allergy Clin Immunol 2004;113:373-9.
11. Björksten B, Dumitrascu D, Foucard T, Khetsuriani N, Khaitov R, Leja M, et al. Prevalence of childhood asthma, rhinitis and eczema in Scandinavia and Eastern Europe. Eur Respir J 1998;12:432-7.
12. Kabesch M, Lauener RP. Why Old McDonald had a farm but no allergies: genes, environment, and the hygiene hypothesis. J Leukoc Biol 2004;75:383-7.
13. Rook GAW, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunopathol 2004;25:237-55.
14. Zuany-Amorim C, Manlius C, Trifilieff A, Brunet LR, Rook G, Bowen G, et al. Long-term protective and antigen-specific effect of heat-killed *Mycobacterium vaccae* in a murine model of allergic pulmonary inflammation. J Immunol 2002;169:1492-9.
15. Adams VC, Hunt JR, Martinelli R, Palmer R, Rook GA, Brunet LR. *Mycobacterium vaccae* induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. Eur J Immunol 2004;34:631-8.

16. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, et al. Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet 2000;356:1723-7.
17. Yazdanbakhsh M, Kremsner P, van Ree R. Allergy, parasites and the hygiene hypothesis. Science 2002;296:490-4.
18. Smits H, Engering A, van der Kleij D, de Jong E, Schipper K, van Capel T, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 2005;115:1260-7.
19. Smit JJ, Folkerts G, Nijkamp FP. Mycobacteria, genes and the 'hygiene hypothesis'. Curr Opin Allergy Clin Immunol 2004;4:57-62.
20. Horner AA, Redecke V, Raz E. Toll-like receptor ligands: hygiene, atopy and therapeutic implications. Curr Opin Allergy Clin Immunol 2004;4:555-61.
21. LeSouëf P, Goldblatt J, Lynch N. Evolutionary adaptation of inflammatory immune responses in human beings. Lancet 2000;356:242-4.
22. Hauswirth DW, Sundy JS. Bioaerosols and innate immune responses in airway diseases. Curr Opin Allergy Clin Immunol 2004;4:361-6.
23. Braun-Fahrländer C. Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. Curr Opin Allergy Clin Immunol 2003;3:325-9.
24. Braun-Fahrländer C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, et al. Prevalence of hay fever and allergic sensitization in farmers' children and their peers living in the same rural community. Clin Exp Allergy 1999;29:28-34.
25. von Ehrenstein O, von Mutius E, Illi S, Bauman L, Böhm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy 2000;30:187-93.
26. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-33.
27. Waser M, von Mutius E, Riedler J, Nowak D, Maisch S, Carr D, et al. Exposure to pets, and the association with hay fever, asthma, and atopic sensitization in rural children. Allergy 2005;60:177-84.
28. Remes S, Iivanainen K, Koskela H, Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? Clin Exp Allergy 2003;33:427-34.
29. von Hertzen L, Mäkelä MJ, Petäys T, Jousilahti P, Kosunen TU, Laatikainen T, et al. Growing disparities in atopy between the Finns and the Russians—a comparison of two generations. J Allergy Clin Immunol 2006;117:151-7.
30. Viinanen A, Munhbayarlah S, Zevgee T, Narantsetseg L, Naidansuren T, Koskenvuo M, et al. Prevalence of asthma, allergic rhinoconjunctivitis and allergic sensitisation in Mongolia. Allergy 2005;60: 1370-7.
31. Yemaneberahn H, Bekele Z, Venn A, Lewis S, Parry E, Britton J. Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. Lancet 1997;350:85-90.
32. Perzanowski M, Ng'ang'a L, Carter M, Odhiambo J, Ngari P, Vaughan J, et al. Atopy, asthma and antibodies to *Ascaris* among rural and urban children in Kenya. J Pediatr 2002;140:582-8.
33. Braun-Fahrländer C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869-77.
34. Nilsson L, Castor O, Löfman O, Magnusson A, Kjellman NI. Allergic disease in teenagers in relation to urban or rural residence at various stages of childhood. Allergy 1999;54:716-21.
35. Viinanen A. Occurrence and risk factors of asthma, allergic rhinoconjunctivitis and allergic sensitisation in rural and urban Mongolia [thesis]. Turku (Finland): University of Turku; 2004. Annales Universitatis Turkuensis D/604.
36. Kalyoncu AF, Stålenheim G. Serum IgE levels and allergic spectra in immigrants to Sweden. Allergy 1992;47:277-80.
37. Grüber C, Illi S, Plieth A, Sommerfeld C, Wahn U. Cultural adaptation is associated with atopy and wheezing among children of Turkish origin living in Germany. Clin Exp Allergy 2002;32:526-31.
38. Matricardi PM, Yazdanbakhsh M. Mycobacteria and atopy, 6 years later: a fascinating, still unfinished, business. Clin Exp Allergy 2003; 33:717-20.
39. Kemp A, Björksten B. Immune deviation and the hygiene hypothesis: a review of the epidemiological evidence. Pediatr Allergy Immunol 2003; 14:74-80.
40. Linneberg A. Hypothesis: urbanization and the allergy epidemic—a reverse case of immuno-therapy. Allergy 2005;60:538-9.
41. Santamaria J, Toranzos GA. Enteric pathogens and soil: a short review. Int Microbiol 2003;6:5-9.
42. Toivola M. Personal exposure to microbial aerosols [thesis]. Kuopio (Finland): University of Kuopio; 2004. Publications of the National Public Health Institute A13/2004.
43. Brisbon N, Plumb J, Brawer R, Paxman D. The asthma and obesity epidemics: the role played by the built environment—a public health perspective. J Allergy Clin Immunol 2005;115:1024-8.
44. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-20.
45. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.
46. Lindström ES, Bergström AK. Community composition of bacterioplankton and cell transport in lakes in two different drainage areas. Aquat Sci 2005;67:210-9.
47. Haileamlak A, Dagoye D, Williams H, Venn AJ, Hubbard R, Britton J. Early life risk factors for atopic dermatitis in Ethiopian children. J Allergy Clin Immunol 2005;115:370-6.
48. Cooper PJ, Chico ME, Rodriguest LC, Strachan DP, Anderson HR, Rodrigues EA, et al. Risk factors for atopy among school children in a rural area of Latin America. Clin Exp Allergy 2004;34:845-52.
49. Young IM, Crawford JW. Interactions and self-organization in the soil-microbe complex. Science 2004;304:1634-7.
50. Liesack W, Janssen PH, Rainey F, Ward-Rainey N, Stackebrandt E. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 375-7.
51. Gisi U, Schkendel R, Schulin R, Standelmann F, Sticker H. Bodenökologie. Stuttgart: Georg Thieme Verlag; 1997.
52. Garrity GN, editor. Bergey's manual of systematic bacteriology. 2nd ed. New York: Springer-Verlag; 2001.
53. Thorn G. The fungi in soil. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 63-127.
54. Bakken LR. Culturable and nonculturable bacteria in soil. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 47-61.
55. Arkwright PD, David TJ. Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderated-to-severe disease. J Allergy Clin Immunol 2001;107:531-4.
56. Isolauri E. Dietary modification of atopic disease: use of probiotics in the prevention of atopic dermatitis. Curr Allergy Asthma Rep 2004;4:270-5.
57. Kalliomäki M, Isolauri E. Role of intestinal flora in the development of allergy. Curr Opin Allergy Clin Immunol 2003;3:15-20.
58. Ogden NS, Bielory L. Probiotics: a complementary approach in the treatment and prevention of pediatric atopic disease. Curr Opin Allergy Clin Immunol 2005;5:179-84.
59. Stanier RY, Doudoroff M, Adelberg EA. General microbiology. London: The Macmillan Press Ltd; 1972.
60. Andersson AM, Weiss N, Rainey F, Salkinoja-Salonen MS. Dust-borne bacteria in animal sheds, schools and children's day care centres. J Appl Microbiol 1999;86:622-34.
61. Frankland AW, Hughes WH, Gorrild RH. Autogenous bacterial vaccines in the treatment of asthma. BMJ 1955;2:941-4.
62. Matricardi PM, Björksten B, Bonini S, Bousquet J, Djukanovic R, Dreborg S, et al. for the EAACI Task Force 7. Microbial products in allergy prevention and therapy. Position paper. Allergy 2003;58: 461-71.
63. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54:1-13.
64. Heine H, Lien E. Toll-like receptors and their function in innate and adaptive immunity. Int Arch Allergy Immunol 2003;130:180-92.
65. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

66. Netea MG, van der Graaf C, van der Meer JWM, Kullberg BJ. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. J Leukoc Biol 2004;75:749-55.
67. Akira S, Sato S. Toll-like receptors and their signalling mechanisms. Scand J Infect Dis 2003;35:555-62.
68. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. J Allergy Clin Immunol 2002;109:379-92.
69. Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 2000;355:1680-3.
70. Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, et al. Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. J Allergy Clin Immunol 2001;108:847-54.
71. Liu AH, Murphy JR. Hygiene hypothesis: fact or fiction? J Allergy Clin Immunol 2003;111:471-8.
72. Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? Curr Opin Allergy Clin Immunol 2004;4:113-7.
73. Westphal O, Lüderitz O. Chemische Erforschung von Lipopolysacchariden Gram-negativer Bakterien. Angew Chemie 1954;66:407-17.
74. Netea MG, van Deuren M, Kullberg BJ, Cavaillon JM, van der Meer JWM. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? Trends Immunol 2002;23:135-9.
75. Seydel U, Schromm AB, Blunck R, Brandenburg K. Chemical structure, molecular conformation and bioactivity of endotoxins. Chem Immunol 2000;74:5-24.
76. Seydel U, Hawkins L, Schromm AB, Heine H, Scheel O, Koch MH, et al. The generalized endotoxic principle. Eur J Immunol 2003;33:1586-92.
77. Jawetz E, Melnick JL, Adelberg EA. Review of medical microbiology. San Francisco (CA): Lange Medical Publications; 1980.
78. van Strien RT, Engel R, Holst O, Bufe A, Eder W, Waser M, et al. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and is association with respiratory health. J Allergy Clin Immunol 2004;113:860-7.
79. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996;64:1906-12.
80. Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 1992;60:3664-72.
81. Keller R, Keist R, Joller PW. Macrophage response to bacteria and bacterial products: modulation of Fc gamma receptors and secretory and cellular activities. Infect Immun 1994;81:161-6.
82. Suda Y, Tochio H, Kawano K, Takada H, Yoshida Y, Kotani S, et al. Cytokine-inducing glycolipids in the lipoteichoic acid fraction from *Enterococcus hirae* ATCC 9790. FEMS Immunol Med Microbiol 1995;12:97-112.
83. Boneca IG. The role of peptidoglycan in pathogenesis. Curr Opin Microbiol 2005;8:46-53.
84. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.
85. Drutz DJ. Fungal diseases. In: Sites DP, Terr AI, Parslow TG, editors. Basic and clinical immunology. East Norwalk: Appleton & Lange; 1994. p. 649-65.
86. Williams DL. Overview of (1, 3)-beta-D-glucan immunobiology. Mediators Inflamm 1997;6:247-50.
87. Netea MG, van der Graaf C, van der Meer JWM, Kullberg BJ. Recognition of fungal pathogens by Toll-like receptors. Eur J Clin Microbiol Infect Dis 2004;23:672-6.
88. Netea MG, Sutmuller R, Hermann C, van der Graaf CA, van der Meer JW, Adema G, et al. Toll-like receptor 2 inhibits cellular responses against *Candida albicans* through pathways mediated by IL-10 and CD4+CD25+ regulatory T cells. J Immunol 2004;172:3712-8.
89. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med 2003;197:1119-24.
90. Gantner BN, Simmons RM, Canavera SJ, Akirta S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 2003;197:1107-17.
91. Tokunaga T, Yamamoto S, Shimada S, Abe H, Fukuda T, Fujisawa Y, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984;72:955-62.
92. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, et al. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol 1992;36:983-97.
93. Bird AP. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acid Res 1980;8:1499-504.
94. Horner AA, Raz E. Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. J Allergy Clin Immunol 2002;110:706-12.
95. Hussain I, Kline JN. DNA, the immune system, and atopic disease. J Investig Dermatol Symp Proc 2004;9:23-8.
96. Wild JS, Sur S. CpG oligonucleotide modulation of allergic inflammation. Allergy 2001;56:365-76.
97. van Uden J, Raz E. Immunostimulatory DNA and applications to allergic disease. J Allergy Clin Immunol 1999;104:902-10.
98. Kitagi K, Jain VV, Businga TR, Hussain I, Kline JN. Immunomodulatory effects of CpG oligonucleotides on established Th2 responses. Clin Diagn Lab Immunol 2002;9:1260-9.
99. Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol 2000;165:4848-53.
100. Curotto de Lafaille MA, Muriglan S, Sunshine MJ, Lei Y, Kutchukhidze N, Furtado GC, et al. Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. J Exp Med 2001;194:1349-59.
101. Bohle B. CpG motifs as possible adjuvants for the treatment of allergic diseases. Int Arch Allergy Immunol 2002;129:198-203.
102. Roy SR, Schiltz AM, Marotta A, Shen Y, Liu AH. Bacterial DNA in house and farm barn dust. J Allergy Clin Immunol 2003;112:571-8.
103. Chen Y, Lenert P, Weerantna R, McCluskie M, Wu T, Davis HL, et al. Identification of methylated CpG motifs as inhibitors of the immune stimulatory CpG motifs. Gene Ther 2001;8:1023-32.
104. Pisetsky DS, Reich CF. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. Clin Immunol 2000;96:198-204.
105. Takabayashi K, Vibet L, Chisholm D, Zubeldia J, Horner AA. Intranasal immunotherapy is more effective than intradermal immunotherapy for the induction of airway allergen tolerance in Th2-sensitized mice. J Immunol 2003;170:3898-905.
106. Hall G, Houghton CG, Rahbek JU, Lamb JR, Jarman ER. Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production. Vaccine 2003;21:549-61.
107. Hawrylowicz CM, O'Garra AO. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 2005;5:271-83.
108. Akbari O, Stock P, DeKruyff RH, Umetsu DT. Mucosal tolerance and immunity: regulating the development of allergic disease and asthma. Int Arch Allergy Immunol 2003;130:108-18.
109. Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480-7.
110. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253-7.
111. Mills KHG, McGuirk P. Antigen-specific regulatory T cells—their induction and role in infection. Semin Immunol 2004;16:107-17.
112. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, et al. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 2002;8:625-9.
113. Curotto de Lafaille MA, Lafaille JJ. The role of regulatory T cells in allergy. Springer Semin Immunopathol 2004;25:295-310.
114. Lauener RP, Birchier T, Adamski J, Braun-Fahrländer C, Bufe A, Herz U, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet 2002;360:465.
115. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrländer C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004;113:482-8.

116. Raby BA, Klimecki WT, Laprise C, Renaud Y, Faith J, Lemire M, et al. Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. Am J Respir Crit Care Med 2002;166:1449-56.
117. Fageras Bottcher M, Hmani-Aifa M, Lindström A, Jenmalm MC, Mai XM, Nilsson L, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-linked interleukin-12(p70) responses in Swedish children. J Allergy Clin Immunol 2004;114:561-7.
118. Re F, Strominger JL. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. J Immunol 2004;173:7548-55.
119. McGuirk P, Mills KHG. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol 2002;23:450-5.

Disconnection of man and the soil: Reason for the asthma and atopy epidemic?

Leena von Hertzen, PhD, and Tari Haahtela, MD, PhD *Helsinki, Finland*

Intense search has been going on to find factors responsible for the asthma and atopy epidemic in Western societies. Attention has increasingly been devoted to environmental saprophytes, which, in addition to gut commensals, might be the major players in the development and fine tuning of immunologic homeostasis. This review outlines current evidence for the role of environmental saprophytes in the development of atopic disease and considers the consequences of urbanization in reducing contacts with soil microorganisms. The major microbial components that have been shown to possess immunomodulatory capacity and their respective Toll-like receptors are also discussed, as are the possible mechanisms underlying the ability of saprophytes to confer protection against atopic disease. (J Allergy Clin Immunol 2006;117: 334-44.)

Key words: *Allergy, asthma, atopy, hygiene hypothesis, saprophytes, urbanization*

The current asthma and atopy epidemic in Western societies has raised a common concern and questions of factors involved. Although in some countries prevalence rates in atopic diseases appear to have leveled off,[1-4] trends are still on the increase in many other countries.[5,6]

Numerous studies have consistently shown that high asthma and atopy rates are associated with urbanization and Western lifestyle.[7,8] Accumulating data suggest that something that is necessary for the normal maturation of the immune system might be lacking in our affluence.[9] Conversely, farm environment and a more traditional lifestyle in nonaffluent countries appear to confer protection against atopic disease.[8,10,11] Although the ultimate factors responsible for the asthma and atopy epidemic have remained unidentified, a common denominator for both living on a farm and in a nonaffluent environment is the heavy exposure to microorganisms in soil and vegetation.

Most of the microorganisms we encounter do not cause any overt infection but are still recognized by the innate immune system. Microbes in this respect need not be alive because even nonviable microbial components interact with the innate immune system. Persistent and moderate environmental exposure to microbial components might play a decisive role in the normal maturation of the immune system in childhood.[12] It has been proposed that certain microorganisms that have been present throughout the mammalian evolutionary history are recognized by the innate immune system as ''no danger'' signals and thus do not trigger inflammatory responses but instead have the ability to induce tolerance through rapid regulatory T (Treg) cell responses.[13] These organisms include saprophytic mycobacteria, lactobacilli, and some intestinal parasites that are able to elicit Treg cell responses *in vivo*[14-17] and *in vitro*.[18] The list of such microbes will certainly grow in the next few years.

The focus of the research in the context of the hygiene hypothesis has largely shifted from overt infections and the T_H1/T_H2 paradigm to noninfectious organisms, Treg cells, and Toll-like receptors (TLRs), as new data have been accumulated and the paradigm was found to be unable to unambiguously explain some important epidemiologic findings.[19,20] Indeed, diseases of immune dysregulation, including atopic diseases, are now considered to develop, more or less, as a result of failure in Treg cell function.[13] Immune defense mechanisms that evolved during the long history of humankind in a hostile environment appear now to be less appropriate when living in a clean environment.[21]

Abbreviations used
TLR: Toll-like receptor
Treg cell: Regulatory T cell

HYPOTHESIS

In this review we propose a hypothesis that one major factor in the current asthma and atopy epidemic might be the disconnection of man and the soil.

EVIDENCE TO SUPPORT THE HYPOTHESIS

There is abundant literature on adverse respiratory health effects attributable to exposure to environmental

From the Helsinki University Central Hospital, Skin and Allergy Hospital.
Supported by the Academy of Finland (grant no. 201246), by Helsinki University Hospital Grants (no. 2250 and 5201), and by the Finnish Anti-Tuberculosis Association Foundation.
Received for publication October 7, 2005; revised November 3, 2005; accepted for publication November 10, 2005.
Reprint requests: Leena von Hertzen, PhD, HUCH, Skin and Allergy Hospital, PO Box 160, 00029 HUS, Finland. E-mail: leena.vonhertzen@kolumbus.fi.
0091-6749/$32.00

doi:10.1016/j.jaci.2005.11.013

bioparticles (eg, bacteria, molds, and fugal spores).[22] Paradoxically, data are now accumulating to suggest that exposure to microbes in soil and vegetation might be beneficial, even necessary, for the normal maturation of the immune system.

Several lines of evidence indicate that settings associated with high-level exposure to microorganisms in soil are associated with reduced risk for asthma and atopy. Such settings include farm environments, environments in nonaffluent Eastern countries, and rural areas, particularly in developing countries.

Farm environment and atopic disease

More than 30 studies from the last 6 years have consistently shown that children who have lived or are living on a farm are less likely to have atopic disease than their counterparts not living on a farm. The issue of farming and atopic disease has been thoroughly reviewed elsewhere and is not reiterated here.[8,10,23] In many of these studies, the effect of parental farming on the development of atopic disease in the child has been found to be dose dependent,[24-26] and many of these studies have also revealed ''frequent contacts with farm animals'' as one of the major factors responsible for this effect.[25,27,28] However, frequent contacts with farm animals can also be a surrogate marker for exposure to microorganisms in soil and vegetation because farm animals (and pets) are likely to serve as a secondary source of exposure to such microorganisms. In addition, frequent contacts with farm animals could also reflect general activity of the child on the farm. The effect of farming on conferring protection against asthma and atopy might not be restricted to early life only because current parental farming has been found to be an even stronger protective factor than that in early life.[28]

Environments in nonaffluent societies: The effect of traditional lifestyle

Frequent compost and waste handling, wood handling, and animal excreta and manure handling are examples of high-level microbial exposure[22] associated with a traditional lifestyle. Unchlorinated surface water from lakes and rivers might be used as domestic water, untreated waste water might be used for irrigation, and animal excreta might be used as manure. Traditional lifestyle might also be associated with a microbe-rich diet (eg, frequent use of fermented vegetables).[20] We found recently that occurrences of atopy (determined by means of skin prick tests) and atopic diseases were substantially lower among schoolchildren and their mothers in Russian Karelia compared with that seen in their counterparts in North Karelia, Finland, irrespective of the geographic proximity of the areas and similar geoclimatic and vegetative conditions (see Fig E1 in the Online Repository at www.jacionline.org). Analysis of generational differences revealed that in Finland children had higher atopy rates than their mothers, whereas in Russia the opposite trend, children having lower atopy rates than their mothers, emerged. No signs of westernization, with atopy prevalence as a proxy, were yet discernible in Russian Karelia, which was part of the Soviet Union until 1991.[29] The results are in line with those reported earlier from other Eastern countries in transition crisis.[11] The East-West gradient in light of the occurrence of atopic diseases has been thoroughly reviewed.[8]

Rural areas and atopic disease: Evidence from relocation studies

Data both from Western and particularly from developing countries, in which great differences in lifestyle still exist between urban and rural areas, have shown that living in rural areas might confer protection against atopic disease, even in a dose-dependent manner.[30-34] A recent study in Mongolia that compared the occurrence of atopy and allergic disorders in 3 different environments of various degree of urbanization—a city area, rural towns, and villages—found significant increasing trends in the prevalence of allergic rhinitis and atopy, as determined by using skin prick tests, with increasing degree of urbanization.[30] Analysis of the effect of relocation revealed that continuous living in a village since birth was most protective against atopy and allergic rhinitis, whereas those who relocated from villages to towns in adolescence or adulthood acquired allergic conditions at rates approaching those found in subjects who had always lived in towns.[35] The results are in line with those of other migrant studies showing that sensitizaton rates and profiles among immigrants shift along with time, resembling finally those in natives,[36,37] thus supporting the view that there might not be any strictly limited window period in early life during which the individual is susceptible to immunomodulatory effects of the environment; rather, susceptibility to immunomodulation probably continues to adolescence, even to adulthood.[38-40] However, it must be borne in mind that disparities between asthma and atopic conditions in this respect might exist.[10]

Indicators of urbanization and atopic disease

Before urbanization, humans have lived in close contact with soil, either directly or indirectly through food, water, and air,[41] and heavy exposure to environmental microorganisms has occurred through inhalation, ingestion, and skin contact.[20] Inhalation of bioaerosols (composed of microbes and their components, such as products of plants and fecal material from animals) has been considered to represent the major route of exposure.[22] This natural exposure to microbes, particularly in soil, has been dramatically reduced along with urbanization characterized by living in environments covered with asphalt and concrete.

There are no unambiguous and commonly accepted criteria for urbanization. Many of the suggested criteria are based on population density and are not relevant for sparsely populated countries, such as Finland.

We performed time-series analyses of occurrence of atopic diseases and urbanization using the asphalt index (use of asphalt, tons per inhabitant per year, years 1960-1990; The Road Administration, the Ministry of Traffic and Communication, and The Finnish Asphalt

Association. Census statistics; Statistics Finland; http://statfin.stat.fi) and the decreasing proportion of farmers among the population (years 1966-2000, Statistics Finland; http://statfin.stat.fi) as indicators of urbanization here because both are closely related to reduced contacts with soil.

Prevalences of asthma and allergic rhinitis were based on our recent data on occurrence of atopic disease among military conscripts.[6] The database here covered the years 1966 through 2000 and comprised more than 1 million military conscripts aged 18 to 19 years. The men had been examined to establish their fitness for service at the call up. Similar diagnostic codes for asthma and allergic rhinitis have been used throughout the study period on the basis of ICD-8 and ICD-9 in 1966 through 1996 and ICD-10 in 1997 through 2000.

We found that the use of asphalt, which in Finland started at the end of the 1950s and was very modest still in the early 1960s (The Road Administration and The Finnish Asphalt Association, unpublished data), increased 10-fold in 3 decades. A nearly similar increase was also found in asthma prevalence among military conscripts, from 0.3% in 1966 to 2.6% in 1995 during a 30-year period, and the trend was upward for the whole study period (Fig 1, *A*).[6]

Along with urbanization, heavy structural changes have occurred in agriculture and forestry. In Finland, the proportion of farmers among the population has decreased from 17.3% in 1970 to 4.9% in 2000. During the same time, the occurrence of allergic rhinitis, as assessed among young Finnish men, increased almost exponentially, from 0.1% in 1966 to 8.9% in 2000 (Fig 1, *B*).[6] The proportion of population that is continuously in natural connection with soil has thus diminished since the 1960s and will evidently still diminish, whereas the opposite has occurred for the prevalence of allergic rhinitis.

Urbanization can also be characterized by living in apartment houses, which is, similarly to the use of asphalt and decrease in farming occupation, likely to reduce contact with soil. Dwelling type has indeed been shown to affect the magnitude of exposure to microorganisms in the environment. A study among 81 randomly selected teachers showed that both personal exposure to microorganisms (assessed with transportable inhalable aerosol samplers) and microbial concentrations in their homes were higher among persons living in family (single) houses compared with those in apartment houses, and this was considered partly to be due to increased outdoor activities among those living in family houses.[42] A sedentary lifestyle with little outdoor activity might not only be involved in the association between asthma and obesity[43] but can also increase the risk of atopy through reduced exposure to saprophytes in the environment.

Although exposure to pathogens has been found to be inversely associated with atopic diseases[44] and undoubtedly is able to exert immunomodulatory effects in early life, infectious agents might represent only a minimal part of our total exposure to microorganisms. The largely neglected group of saprophytes in the environment might play a decisive role, in addition to gut microbiota,[45] in the development and maintenance of immunologic homeostasis.

Source of drinking water and atopic disease

An important issue closely related to soil is the runoff of soil microorganisms into natural waters[46] and the use of such waters as drinking water. We found that in Russian Karelia, where atopy and atopic diseases are uncommon,[29] surface water bodies, lakes and rivers, are used as domestic water, frequently without any chemical or other treatment. Previous data have shown that consumption of unpasteurized milk in early life is associated with reduced risk of asthma and atopy in later life independently from other determinants.[26] It is reasonable to assume that consumption of untreated surface water could have similar effects and could be involved in the low atopy prevalence in Russian Karelia. Indeed, this view is supported by recent data from Ethiopia showing that consumption of river water in rural areas, as contrasted with consumption of pipe water in urban areas, conferred protection against atopic eczema.[47] In another study among schoolchildren in a rural area of Latin America, consumption of river water was found to be weakly protective against atopy.[48]

SOIL MICROBIOTA

Soil is considered the most complicated biomaterial and at the same time the most diverse and important ecosystem on the planet.[49] The definition of the microbial composition of a typical soil has proved to be problematic because of this diversity of soil types and the complexity and variability of the physicochemical circumstances. Nonetheless, the majority of soil bacteria are considered to belong to the lineage of gram-positive bacteria,[50] and members of the phylum Actinobacter have been found to predominate in the soil.[51] This phylum includes genera such as *Mycobacterium* species, *Streptomyces* species, *Actinomyces* species, *Corynebacterium* species, and *Bifidobacterium* species.[52] Fungi are often dominant in soils in terms of their biomass, particularly fungi dominate in acid temperate or polar soils that are oligotrophic,[53,54] whereas bacteria predominate in near-neutral or moderately alkaline soils.[54] Some estimates of the density of microorganisms in a normal near-neutral organic soil obtained by means of cultivation and microscopy have been reported: Actinobacter, for example, might occur at the concentrations of $10^{9\text{-}13}$ bacteria/dm3 soil and other bacteria at the level of 10^{8}/dm3 soil.[51] Cultivation, which has traditionally been used to measure bacterial densities in soil samples, probably greatly underestimates the true values. The more modern methods, such as PCR tests and fatty acid analyses, have revealed that a considerable proportion of all bacteria in soil is in a dormant (metabolically inactive) stage.

Mycobacteria, one of the major bacterial groups in soil and natural waters, including more than 80 saprophytic species,[13] has received considerable attention during the last decade as a potential immunomodulatory agent in

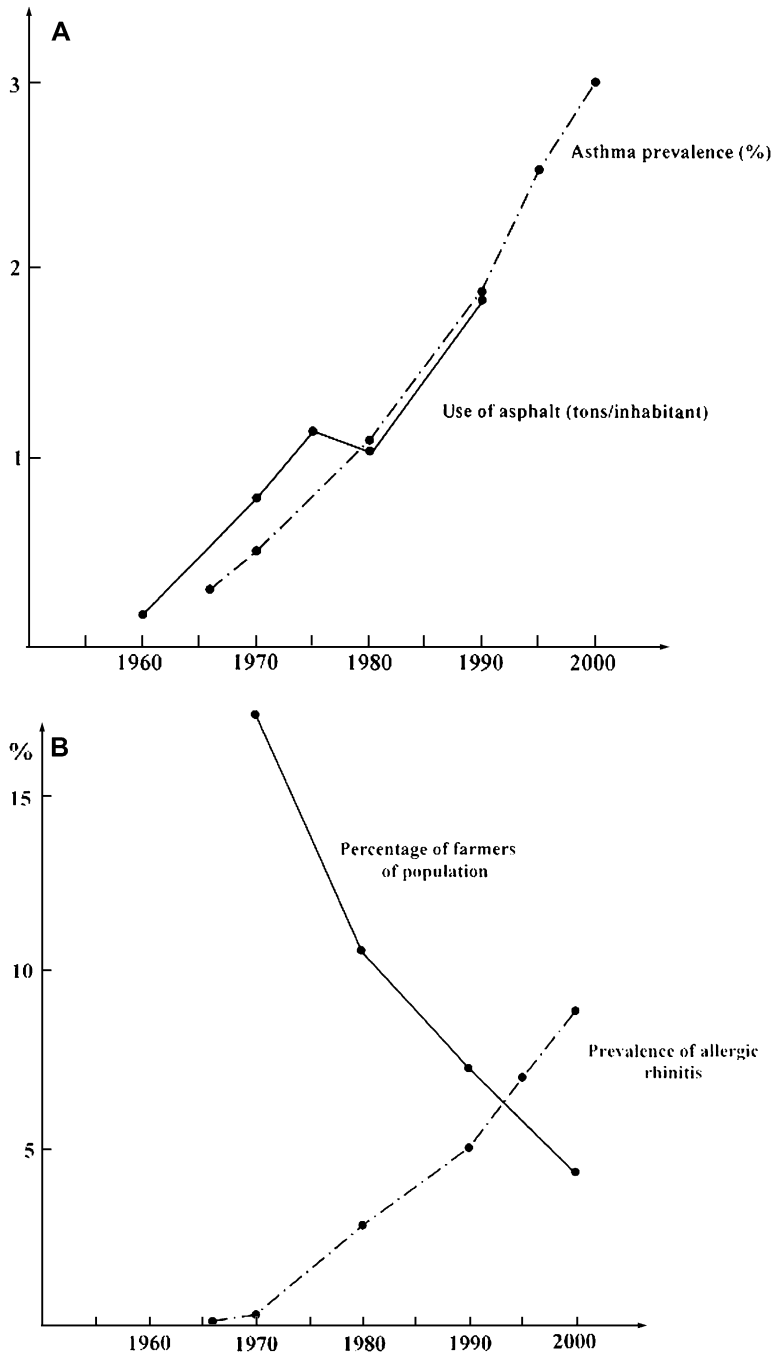


FIG 1. A, Use of asphalt (tons per inhabitant per year) from 1960 through 1990 and occurrence of diagnosed asthma among military conscripts from 1966 through 2000 in Finland.[6] **B**, Proportion of farmers among the whole population from 1970 through 2000 and occurrence of diagnosed allergic rhinitis among military conscripts from 1966 through 2000 in Finland.[6] Supplemental information is available in the Online Repository at www.jacionline.org.

alleviating symptoms of atopic disease[55] and even conferring protection against them.[14,15] In addition, much of the current research of atopic diseases has been focused on lactobacilli, which are considered one of the potential groups of immunomodulatory agents with prophylactic and therapeutic potential.[56-58] Notably, lactobacilli are originally common inhabitants of plants and grow at the expense of the nutrients liberated from decomposing plant material.[59]

Results from our laboratory indicate that, indeed, gram-positive bacteria represent the great majority (90%) of all bacteria in settled barn dust measured by means of

exact fatty acid analyses (Saris et al, unpublished data). However, the proportions of gram-negative and gram-positive bacteria might vary to some extent according to the season,[60] particularly in northern boreal latitudes, where the soil is frozen and covered with snow for months.

MAJOR COMPONENTS OF MICROORGANISMS WITH IMMUNOMODULATORY POTENTIAL AND THEIR INTERACTION WITH THE INNATE IMMUNITY

The idea that microbial products have immunomodulatory potential and could be used as immunotherapeutic agents in asthma and allergies dates back to the 1950s.[61] Bacterial extracts obtained mainly from species associated with upper respiratory tract and urinary tract infections administered subcutaneously were earlier used for such purposes but are not in use today because several double-blind studies showed no efficacy in asthma, possibly because of overly low concentrations of bacterial material in these extracts, nonoptimal route of administration, and overly long intervals between the doses (1 week or longer). No studies on the preventive effect of oral bacterial extracts are available.[62]

Because both viable and nonviable bacterial components have been found to be immunobiologically active, a renewed interest in bacterial cell-wall components has been raised, although the literature on immunologic effects of cell-wall components other than LPS (endotoxin) is relatively scarce. However, in addition to LPS, 2 other ubiquitous bacterial components, lipoteichoic acids and peptidoglycans, might be of importance in this respect, and the cell-wall components of fungi (eg, β-glucans) have also been found to have immunomodulatory potential. In addition, a potent immunomodulator appears to be bacterial DNA, the unmethylated CpG oligonucleotide.

TLRs

TLRs, the receptors that recognize conserved microbial structures, represent an ancient system of host defense but were not discovered until 1989.[63,64] This discovery brought the formerly underappreciated innate immunity into the focus of research. TLRs have been extensively reviewed during the last few years.[64-67] To date, at least 10 different TLRs have been identified in mammals, and they have been found to play a decisive role in recognizing microbes and bridging the innate and acquired immune responses.[66] Although the role of TLRs has been mostly shown in infectious systems, there are good reasons to believe that TLRs are equally important in repeated exposure to saprophytic bacteria in the environment and commensals in the gut and are involved in the induction of tolerance. TLR2, TLR4, and TLR9 are briefly considered here in the light of exposure to environmental saprophytes. TLR2 is the principal receptor and signaling molecule for gram-positive bacteria, lipoteichoic acid, mycobacteria, mycobacterial lipoarabinomannan, bacterial lipoproteins, and fungal β-glucan. TLR4 is the primary receptor for LPS from gram-negative bacteria, and TLR9 recognizes bacterial unmethylated CpG oligonucleotides.[64]

Major cell-wall components of bacteria and fungi

LPS (endotoxin). The chemical composition of LPS, the major cell-wall component of gram-negative bacteria, has been known for more than 50 years, and its physicochemical properties, stability and heat resistance, are also well established.[68] Abundant literature exists concerning the biologic effects of this macromolecule. In several studies the relative lack of exposure to endotoxin has been suggested to be one major reason for the asthma and atopy epidemic.[33,69,70] The relationship between LPS and the occurrence of asthma and atopy has also been thoroughly reviewed elsewhere.[68,71,72]

It has also been known since the 1950s that the biologic activity of LPS resides in the lipid A moiety (the theory of the ''endotoxic principle''[73]). Moreover, it is now well established that the immune response to LPS is dependent on the chemical structure and molecular conformation of the lipid A moiety. Lipid A consists of a phosphorylated glucosamine disaccharide (the backbone), to which fatty acids are attached. It has been found that biologic effects of LPS from different gram-negative bacteria are not similar.[74] The critical determinants here are the length and number of acyl (fatty acid) chains, the asymmetry of these chains, and the number and distribution of negative charges.[74,75] Interestingly, the widely used test to detect LPS in various samples, the Limulus amebocyte lysate test, has been found to be specific for the LPS glucosamine backbone and is thus not a measure of biologic activity. Fatty acid analyses might better correlate with biologic activity because the conformation and number of acyl (fatty acid) chains in lipid A appear to be central to the capacity of LPS to interact with TLRs and to induce cytokine production.[76]

Peptidoglycan and teichoic acid. With the exception of a few groups of some minor bacteria, such as *Mycoplasma* and *Chlamydia* species, all members in the domain Bacteria have one common denominator, the presence of peptidoglycan as the main strengthening and shape-determining constituent of the cell wall. In gram-positive bacteria, peptidoglycan accounts for at least 50%, and often more, of the total dry weight of the wall, but in gram-negative bacteria, it might comprise less than 10% of the dry weight of the wall.[59] In gram-negative bacteria peptidoglycan is localized in the innermost layer of the wall and, although extremely thin, is still capable of retaining the shape of the cell.[59] Peptidoglycan is composed of 2 amino sugars, N-acetylglucosamine and N-acetylmuramic acid, and a side chain of 4 amino acids that can vary from species to species.[77] The other of these amino sugars, N-acetylmuramic acid, is a molecule specific to the domain Bacteria and can thus be used for laboratory diagnostic purposes. In a recent study, van Strien et al[78] showed that muramic acid can be found in dust from children's mattresses and in higher concentrations in those of farmer's children.

Wheezing and asthma were inversely associated with the muramic acid concentration, independently from LPS, whereas no association was found for atopic sensitization. Increased muramic acid concentrations were found in homes heated with wood or coal, independent of whether it was a farm home, which suggests that settings associated with traditional lifestyle and increased exposure to microorganisms, rather than farming per se, is responsible for this effect.

The other characteristic cell-wall components of most gram-positive bacteria are teichoic acids (up to 50% of the dry weight of the wall). Teichoic acids are water-soluble polymers containing ribitol or glycerol residues joined through phosphodiester linkages.[77] In lipoteichoic acids there is a single lipid side chain anchored to the ribitol or glycerol backbone.[79] Teichoic and lipoteichoic acids have been found to exert potent inflammatory responses.[80-82] As stated above, teichoic acids are recognized by TLR2. Peptidoglycan has similarly been considered to be recognized by TLR2,[64,67] but this view has recently been challenged by the identification of intracellular proteins, the nucleotide-binding oligomerization domain 1 and 2, as the principal receptors for peptidoglycan fragments.[83,84]

Fungal cell-wall components. Fungal cell walls differ from those of bacteria by lacking peptidoglycan, teichoic acids, and LPS. In their place are the external and antigenic peptidomannans embedded in matrices of α- and β-glucans, and structural rigidity is provided by chitin.[85] The principal sterol in fungal cell membranes is ergosterol (corresponding cholesterol in mammalian cells), which has been used, in addition to β-glucans, in laboratory diagnostics of environmental samples. The immunobiology of β-glucans has been reviewed earlier.[86] Most of the recent studies on fungal recognition by TLRs have been centered on a few potentially pathogenic fungi, such as *Candida albicans* and *Aspergillus fumigatus*. It has been found that both TLR2 and TLR4 can be important for their recognition[87] and that TLR2 might be involved in maintaining prolonged candidasis by mediating anti-inflammatory signals leading to IL-10 production and generation of regulatory T cells.[88] In addition, a new coreceptor for β-glucan, dectin-1, expressed on macrophages, dendritic cells, and monocytes, has been found to be involved in mediating proinflammatory responses to fungi together with TLR2.[89,90] The recognition of fungi by the innate immune system appears to be more complex than that of bacteria because fungi can exist in 2 forms, as hyphae or conidiae.

Bacterial CpG oligonucleotides

Since the late 1980s, bacterial DNA has been known to possess immunostimulatory properties[91] that are not found in vertebrate DNA.[92] The activating element was identified as an unmethylated CpG oligonucleotide that was found to be 20-fold more abundant in bacterial than in vertebrate DNA, and when present in vertebrate DNA, about 70% of it was found to be methylated.[93]

Unmethylated CpG oligonucleotides have the ability to elicit a multifaceted innate immune response characterized by the production of IL-12, IL-18, and IFN-γ and the upregulation of costimulatory molecules by antigen-presenting cells, B cells, and natural killer cells.[94] They have both direct and indirect effects on the commitment of $CD4^+$ cells to a T_H1 phenotype and are thus able to downregulate or reverse T_H2 responses.[94-96] Mammalian DNA or methylated bacterial DNA, in contrast, does not induce these responses.[97] In addition, CpG oligonucleotides have been found to strongly induce IL-10 release, which inhibits both T_H1 and T_H2 responses in a dose-dependent manner.[98] This IL-10 is the key cytokine in the development of adaptive regulatory T (Treg) cells, which in turn are able to downregulate antigen-specific IgE responses and promote tolerance to allergens.[99,100] Data on synthetic CpG oligonucleotides in murine models of atopic diseases and as vaccine adjuvants and therapeutic agents in human subjects with allergic disorders are promising.[94-96,101]

In a study by Roy et al,[102] bacterial DNA and LPS contents in dust from urban, rural, and farm homes and from farm barns were quantified (by means of PCR specific for bacterial ribosomal DNA and the Limulus test, respectively) to determine whether there are differences in the immune stimulatory capacity between different dust samples. The highest bacterial DNA levels were found in farm barns, followed by rural homes, farm homes, and urban homes. Farm barn DNA significantly potentiated LPS-induced IL-10 and IL-12p40 release from PBMCs, whereas DNA from urban homes did not show this effect, probably because of the low content of bacterial DNA in urban home dust; only approximately 3% of the total DNA content in urban samples was bacterial in origin. Increased IL-10 and IL-12 release shown after stimulation of PBMCs with barn dust DNA and LPS might be crucial in the context of environments conferring protection against atopic diseases.[102] Furthermore, we found a 3.5-fold higher bacterial DNA content (measured by means of bacterial ribosome-specific PCR) in barn dust compared with urban-suburban home dust (5127 vs 1479.5 ng bacterial DNA/g dust; Saris et al, unpublished data). It is known that vertebrate DNA does not possess immunostimulatory capacity but might neutralize or even inhibit the immunostimulatory effects of bacterial CpG motifs.[103,104] The only known TLR for unmethylated CpG oligonucleotides is TLR9.

The innate immunity recognizes saprophytic bacteria, which results in release of proinflammatory cytokines. We have shown *in vitro* that robust responses are elicited in murine macrophages when they are stimulated by common soil microorganisms isolated from barn dust, such as *Streptomyces* species, *Sphingomonas* species, and *Macrococcus* species (Pylkkänen et al, unpublished data), and a dose-dependent and rather similar response was found in the production of, for example, TNF-α for all 3 organisms, contrary to *Bacillus* species, which showed minimal response in this *in vitro* setting (Pylkkänen et al, unpublished data). None of these genera represents true gram-negative bacteria, because *Sphingomonas* species, although categorized as gram-negative, do not possess

LPS but have sphingolipids instead. Interestingly, we found that the dominant (>85%) bacterial genus in dust from urban-suburban homes was *Bacillus* species (Saris and Andersson, unpublished data), lending further support to the view that urban home dust might have minimal, if any, immunomodulatory capacity. Nonetheless, the ability of the common soil saprophytes *Streptomyces* species, *Sphingomonas* species, and *Macrococcus* species to elicit robust proinflammatory cytokine responses *in vitro* raises the question of tolerance, which must have evolved during the long history of coexistence of these saprophytes and man.[13]

TOLERANCE AND Treg CELLS

Repeated or persistent exposure appears to be one fundamental factor in the induction of tolerance. Repeated intranasal antigen exposure leads to decreased bronchial reactivity and tolerance in T_H2-sensitized mice.[105,106] In addition, allergen desensitization therapy (injection of a specific allergen extract at increasing doses) has for years been successfully used, particularly in patients with hay fever and insect venom allergy.[107] Tolerance is mediated by several mechanisms, including anergy and deletion of effector T-cell clones, and particularly by the induction and function of Treg cells.[108] These Treg cells are defined as cells that actively control the function of other cells, mostly in an inhibitory way.[109] Two major lineages of Treg cells have been identified: (1) naturally occurring, thymus-derived Treg cells expressing the transcription factor Foxp3, which are associated primarily with the control of autoantigens, and (2) induced (adaptive), antigen-specific Treg cells, which require IL-10, TGF-β, or both for their differentiation and function.[107,110] These cells ameliorate inflammation through the release of IL-10, TGF-β, or both in repeated or persistent exposure to prevent immune pathology (a form of tolerance) and maintain the persistence of low numbers of antigens in the body, which is necessary in certain cases to provide long-lasting immunity against reinfections.[111] Induction of antigen-specific Treg cells has been performed by administration of heat-killed *Mycobacterium vaccae* and allergen (ovalbumin) into mice. These specific Treg cells were found to release IL-10 and TGF-β and suppress eosinophilia and bronchial hyperresponsiveness.[112] Desensitization therapy has also been found to operate through IL-10.[107] In general, Treg cells are able to prevent the development of highly polarized T_H cells,[113] and one of the mechanisms involved in the development of asthma and atopy has been suggested to be a failure in Treg cell function.[107,109,113] Several excellent review articles of Treg cells are available for further reading.[107,109,110,113]

Treg cells can be preferentially induced at mucosal surfaces, particularly in the gut and respiratory tract.[111] In urbanized Western societies the natural environment might no longer have the ability to maintain the respiratory and gut mucosal system in a state that favors the development of Treg cells and mucosal tolerance to harmless bioparticles.[109] Persistent exposure to saprophytic bacteria in soil and vegetation, in addition to commensals in the gut and respiratory tract, might be needed to stimulate the production of IL-10 and TGF-β through the innate immune system, which in turn are required for the development of inducible Treg cells.

TLRs AND GENE-ENVIRONMENT INTERACTION

Innate immunity is now recognized as a central element also in the gene-environment interaction. The significance of particularly the TLR2 gene in this respect has been demonstrated in several studies. In European children TLR2 gene expression has been found to be higher in blood cells obtained from farmers' children compared with that seen in children not growing up on a farm. A similar difference was not found for the TLR4 gene.[114] Furthermore, a genetic variation in the TLR2 gene was shown to be a major determinant of reduced susceptibility to asthma and atopy in farmers' children but not in nonfarmers' children.[115] No clear association could be found between variations in the TLR4 gene and asthma or hay fever either in farmers' or nonfarmers children in most,[115,116] albeit not all,[117] studies.

These studies provide convincing evidence for gene-environment interactions: a certain polymorphism is expressed only in a certain environment. They also underscore the significance of TLR2 in environments associated with high exposure to soil microorganisms. Because TLR2 is the main receptor for gram-positive bacteria and their structural molecules, lipoteichoic acid and lipoproteins,[64,67] these studies might also point to the significance of particularly gram-positive bacteria in this context. Interaction of TLR2 with its ligands has been found to lead to rapid release of IL-10, which can block the induction of IL-12p35 and IFN-γ by TLR3 and TLR4.[118] It remains to be clarified whether TLR2 is the crucial Toll receptor in mediating IL-10 release for the development of inducible Treg cells.

CONCLUDING REMARKS

Several lines of evidence support the view that the environment in modern industrialized societies is unable to provide the stimulation for the developing immune system that might be beneficial or even necessary: disruption of the ancient connection of humankind and the soil might have had unexpected consequences.

The immunomodulatory role of saprophytic bacteria in soil and vegetation is now increasingly recognized. The innate immune system recognizes such saprophytes or their nonviable components encountered at respiratory and gut mucosal surfaces; however, robust inflammatory responses are not normally elicited but kept in tight control through mechanisms that involve the function of Treg cells that in turn might control the development of atopic

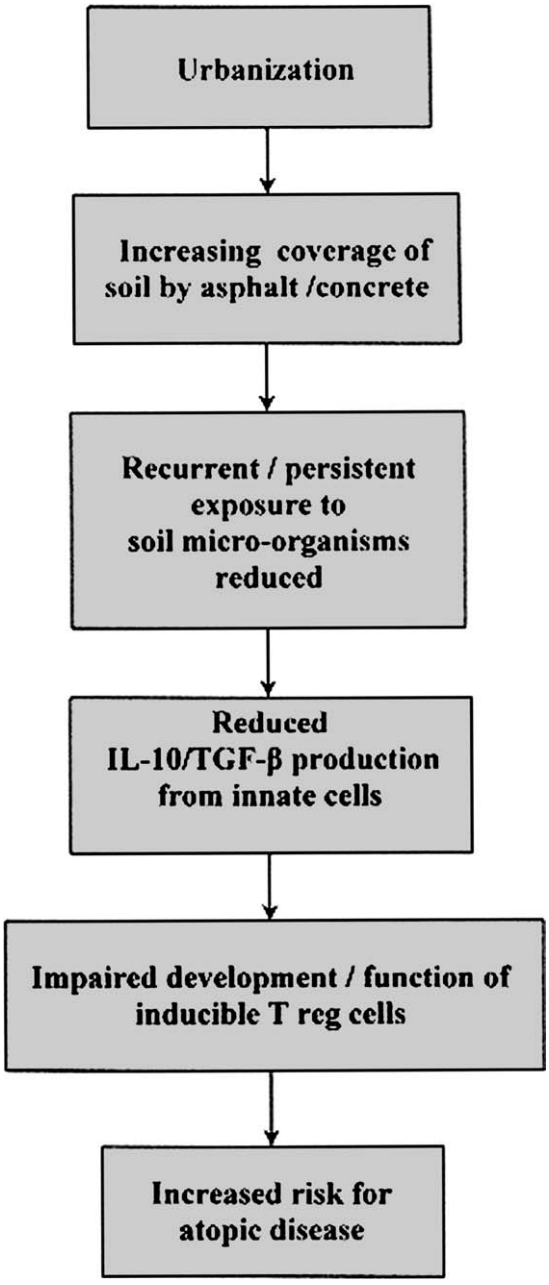


FIG 2. A model of the asphalt theory in the development of atopic diseases. Supplemental information is available in the Online Repository at www.jacionline.org. Because of the implementation of new technologies, including recycling of asphalt material, the figures for the use of asphalt from 1991 onward are not comparable with the earlier ones and have not been included in the analysis. Figures for the years 1960 through 1974 are based on unofficial data from the Road Administration, Ministry of Traffic and Communication, because no official statistics are available for that period. Data on the prevalence of diagnosed asthma and allergic rhinitis are based on Latvala et al.[6]

diseases. A number of microorganisms and their components have been found to induce the production of IL-10, the differentiation factor of inducible Treg cells, by innate cells.[111,119]

Here we proposed a hypothesis that the disconnection of man and the soil might be one major factor in the current asthma and atopy epidemic, as shown in Fig 2. The hypothesis could be tested in animal models and in further comparative and more clearly defined epidemiologic settings, and if correct, a strategy that involves enhancement of the development and activity of Treg cells[117] without concomitant induction of inflammation by bacterial products is evidently the goal to pursue. Central to the outcome of this strategy is probably timing (with respect to the primary sensitization), duration, dose, and route of exposure. The nature of the microbe might also play a role, although it is likely that there is no single agent or agent group behind the protective effect; rather, a mixture, including members of the phylum Actinobacter, might be involved. In addition, the product might not be based on bacterial cell-wall components only because bacterial DNA might have beneficial effects in this respect.

REFERENCES

1. Braun-Fahrländer C, Gassner M, Grize L, Takken-Sahli K, Neu U, Sticker T, et al. No further increase in asthma, hay fever and atopic sensitisaton in adolescents living in Switzerland. Eur Respir J 2004;23: 407-13.
2. Ronchetti R, Villa MP, Barreto M, Rota R, Pagani J, Martella S, et al. Is the increase in childhood asthma coming to an end? Findings from three surveys of schoolchildren in Rome, Italy. Eur Respir J 2001;17:881-6.
3. Verlato G, Corsico A, Villani S, Cerveri I, Migliore E, Accordini S, et al. Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study. J Allergy Clin Immunol 2003;111: 1232-8.
4. Yura A, Shimizu T. Trends in the prevalence of atopic dermatitis in school children: longitudinal study in Osaka Prefecture, Japan, from 1985 to 1997. Br J Dermatol 2001;145:966-73.
5. Bråbäck L, Hjern A, Rasmussen F. Trends in asthma, allergic rhinitis and eczema among Swedish conscripts from farming and non-farming environments. A nationwide study over three decades. Clin Exp Allergy 2004;34:38-43.
6. Latvala J, von Hertzen L, Lindholm H, Haahtela T. Trends in prevalence of asthma and allergy in Finnish young men: a nationwide study from 1966 to 2003. BMJ 2005;330:1186-7.
7. von Mutius E. The environmental predictors of allergic disease. J Allergy Clin Immunol 2000;105:9-19.
8. von Hertzen L, Haahtela T. Asthma and atopy—the price of affluence? Allergy 2004;59:124-37.
9. Ring J, Krämer U, Schäfer T, Behrendt H. Why are allergies increasing? Curr Opin Immunol 2001;13:701-8.
10. von Mutius E. Influences in allergy: epidemiology and the environment. J Allergy Clin Immunol 2004;113:373-9.
11. Björksten B, Dumitrascu D, Foucard T, Khetsuriani N, Khaitov R, Leja M, et al. Prevalence of childhood asthma, rhinitis and eczema in Scandinavia and Eastern Europe. Eur Respir J 1998;12:432-7.
12. Kabesch M, Lauener RP. Why Old McDonald had a farm but no allergies: genes, environment, and the hygiene hypothesis. J Leukoc Biol 2004;75:383-7.
13. Rook GAW, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunopathol 2004;25:237-55.
14. Zuany-Amorim C, Manlius C, Trifilieff A, Brunet LR, Rook G, Bowen G, et al. Long-term protective and antigen-specific effect of heat-killed *Mycobacterium vaccae* in a murine model of allergic pulmonary inflammation. J Immunol 2002;169:1492-9.
15. Adams VC, Hunt JR, Martinelli R, Palmer R, Rook GA, Brunet LR. *Mycobacterium vaccae* induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. Eur J Immunol 2004;34:631-8.

16. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, et al. Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet 2000;356:1723-7.
17. Yazdanbakhsh M, Kremsner P, van Ree R. Allergy, parasites and the hygiene hypothesis. Science 2002;296:490-4.
18. Smits H, Engering A, van der Kleij D, de Jong E, Schipper K, van Capel T, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 2005;115:1260-7.
19. Smit JJ, Folkerts G, Nijkamp FP. Mycobacteria, genes and the 'hygiene hypothesis'. Curr Opin Allergy Clin Immunol 2004;4:57-62.
20. Horner AA, Redecke V, Raz E. Toll-like receptor ligands: hygiene, atopy and therapeutic implications. Curr Opin Allergy Clin Immunol 2004;4:555-61.
21. LeSouëf P, Goldblatt J, Lynch N. Evolutionary adaptation of inflammatory immune responses in human beings. Lancet 2000;356:242-4.
22. Hauswirth DW, Sundy JS. Bioaerosols and innate immune responses in airway diseases. Curr Opin Allergy Clin Immunol 2004;4:361-6.
23. Braun-Fahrländer C. Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. Curr Opin Allergy Clin Immunol 2003;3:325-9.
24. Braun-Fahrländer C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, et al. Prevalence of hay fever and allergic sensitization in farmers' children and their peers living in the same rural community. Clin Exp Allergy 1999;29:28-34.
25. von Ehrenstein O, von Mutius E, Illi S, Bauman L, Böhm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy 2000;30:187-93.
26. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-33.
27. Waser M, von Mutius E, Riedler J, Nowak D, Maisch S, Carr D, et al. Exposure to pets, and the association with hay fever, asthma, and atopic sensitization in rural children. Allergy 2005;60:177-84.
28. Remes S, Iivanainen K, Koskela H, Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? Clin Exp Allergy 2003;33:427-34.
29. von Hertzen L, Mäkelä MJ, Petäys T, Jousilahti P, Kosunen TU, Laatikainen T, et al. Growing disparities in atopy between the Finns and the Russians—a comparison of two generations. J Allergy Clin Immunol 2006;117:151-7.
30. Viinanen A, Munhbayarlah S, Zevgee T, Narantsetseg L, Naidansuren T, Koskenvuo M, et al. Prevalence of asthma, allergic rhinoconjunctivitis and allergic sensitisation in Mongolia. Allergy 2005;60: 1370-7.
31. Yemaneberahn H, Bekele Z, Venn A, Lewis S, Parry E, Britton J. Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. Lancet 1997;350:85-90.
32. Perzanowski M, Ng'ang'a L, Carter M, Odhiambo J, Ngari P, Vaughan J, et al. Atopy, asthma and antibodies to *Ascaris* among rural and urban children in Kenya. J Pediatr 2002;140:582-8.
33. Braun-Fahrländer C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869-77.
34. Nilsson L, Castor O, Löfman O, Magnusson A, Kjellman NI. Allergic disease in teenagers in relation to urban or rural residence at various stages of childhood. Allergy 1999;54:716-21.
35. Viinanen A. Occurrence and risk factors of asthma, allergic rhinoconjunctivitis and allergic sensitisation in rural and urban Mongolia [thesis]. Turku (Finland): University of Turku; 2004. Annales Universitatis Turkuensis D/604.
36. Kalyoncu AF, Stålenheim G. Serum IgE levels and allergic spectra in immigrants to Sweden. Allergy 1992;47:277-80.
37. Grüber C, Illi S, Plieth A, Sommerfeld C, Wahn U. Cultural adaptation is associated with atopy and wheezing among children of Turkish origin living in Germany. Clin Exp Allergy 2002;32:526-31.
38. Matricardi PM, Yazdanbakhsh M. Mycobacteria and atopy, 6 years later: a fascinating, still unfinished, business. Clin Exp Allergy 2003; 33:717-20.
39. Kemp A, Björksten B. Immune deviation and the hygiene hypothesis: a review of the epidemiological evidence. Pediatr Allergy Immunol 2003; 14:74-80.
40. Linneberg A. Hypothesis: urbanization and the allergy epidemic—a reverse case of immuno-therapy. Allergy 2005;60:538-9.
41. Santamaria J, Toranzos GA. Enteric pathogens and soil: a short review. Int Microbiol 2003;6:5-9.
42. Toivola M. Personal exposure to microbial aerosols [thesis]. Kuopio (Finland): University of Kuopio; 2004. Publications of the National Public Health Institute A13/2004.
43. Brisbon N, Plumb J, Brawer R, Paxman D. The asthma and obesity epidemics: the role played by the built environment—a public health perspective. J Allergy Clin Immunol 2005;115:1024-8.
44. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-20.
45. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.
46. Lindström ES, Bergström AK. Community composition of bacterioplankton and cell transport in lakes in two different drainage areas. Aquat Sci 2005;67:210-9.
47. Haileamlak A, Dagoye D, Williams H, Venn AJ, Hubbard R, Britton J. Early life risk factors for atopic dermatitis in Ethiopian children. J Allergy Clin Immunol 2005;115:370-6.
48. Cooper PJ, Chico ME, Rodriguest LC, Strachan DP, Anderson HR, Rodrigues EA, et al. Risk factors for atopy among school children in a rural area of Latin America. Clin Exp Allergy 2004;34:845-52.
49. Young IM, Crawford JW. Interactions and self-organization in the soil-microbe complex. Science 2004;304:1634-7.
50. Liesack W, Janssen PH, Rainey F, Ward-Rainey N, Stackebrandt E. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 375-7.
51. Gisi U, Schkendel R, Schulin R, Standelmann F, Sticker H. Bodenökologie. Stuttgart: Georg Thieme Verlag; 1997.
52. Garrity GN, editor. Bergey's manual of systematic bacteriology. 2nd ed. New York: Springer-Verlag; 2001.
53. Thorn G. The fungi in soil. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 63-127.
54. Bakken LR. Culturable and nonculturable bacteria in soil. In: van Elsas JD, Trevors JT, Wellington EM, editors. Modern soil microbiology. New York: Marcel Dekker, Inc; 1997. p. 47-61.
55. Arkwright PD, David TJ. Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderated-to-severe disease. J Allergy Clin Immunol 2001;107:531-4.
56. Isolauri E. Dietary modification of atopic disease: use of probiotics in the prevention of atopic dermatitis. Curr Allergy Asthma Rep 2004;4:270-5.
57. Kalliomäki M, Isolauri E. Role of intestinal flora in the development of allergy. Curr Opin Allergy Clin Immunol 2003;3:15-20.
58. Ogden NS, Bielory L. Probiotics: a complementary approach in the treatment and prevention of pediatric atopic disease. Curr Opin Allergy Clin Immunol 2005;5:179-84.
59. Stanier RY, Doudoroff M, Adelberg EA. General microbiology. London: The Macmillan Press Ltd; 1972.
60. Andersson AM, Weiss N, Rainey F, Salkinoja-Salonen MS. Dust-borne bacteria in animal sheds, schools and children's day care centres. J Appl Microbiol 1999;86:622-34.
61. Frankland AW, Hughes WH, Gorrild RH. Autogenous bacterial vaccines in the treatment of asthma. BMJ 1955;2:941-4.
62. Matricardi PM, Björksten B, Bonini S, Bousquet J, Djukanovic R, Dreborg S, et al. for the EAACI Task Force 7. Microbial products in allergy prevention and therapy. Position paper. Allergy 2003;58: 461-71.
63. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54:1-13.
64. Heine H, Lien E. Toll-like receptors and their function in innate and adaptive immunity. Int Arch Allergy Immunol 2003;130:180-92.
65. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

66. Netea MG, van der Graaf C, van der Meer JWM, Kullberg BJ. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. J Leukoc Biol 2004;75:749-55.
67. Akira S, Sato S. Toll-like receptors and their signalling mechanisms. Scand J Infect Dis 2003;35:555-62.
68. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. J Allergy Clin Immunol 2002;109:379-92.
69. Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 2000;355:1680-3.
70. Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, et al. Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. J Allergy Clin Immunol 2001;108:847-54.
71. Liu AH, Murphy JR. Hygiene hypothesis: fact or fiction? J Allergy Clin Immunol 2003;111:471-8.
72. Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? Curr Opin Allergy Clin Immunol 2004;4:113-7.
73. Westphal O, Lüderitz O. Chemische Erforschung von Lipopolysacchariden Gram-negativer Bakterien. Angew Chemie 1954;66:407-17.
74. Netea MG, van Deuren M, Kullberg BJ, Cavaillon JM, van der Meer JWM. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? Trends Immunol 2002;23:135-9.
75. Seydel U, Schromm AB, Blunck R, Brandenburg K. Chemical structure, molecular conformation and bioactivity of endotoxins. Chem Immunol 2000;74:5-24.
76. Seydel U, Hawkins L, Schromm AB, Heine H, Scheel O, Koch MH, et al. The generalized endotoxic principle. Eur J Immunol 2003;33:1586-92.
77. Jawetz E, Melnick JL, Adelberg EA. Review of medical microbiology. San Francisco (CA): Lange Medical Publications; 1980.
78. van Strien RT, Engel R, Holst O, Bufe A, Eder W, Waser M, et al. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and is association with respiratory health. J Allergy Clin Immunol 2004;113:860-7.
79. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996;64:1906-12.
80. Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 1992;60:3664-72.
81. Keller R, Keist R, Joller PW. Macrophage response to bacteria and bacterial products: modulation of Fc gamma receptors and secretory and cellular activities. Infect Immun 1994;81:161-6.
82. Suda Y, Tochio H, Kawano K, Takada H, Yoshida Y, Kotani S, et al. Cytokine-inducing glycolipids in the lipoteichoic acid fraction from *Enterococcus hirae* ATCC 9790. FEMS Immunol Med Microbiol 1995;12:97-112.
83. Boneca IG. The role of peptidoglycan in pathogenesis. Curr Opin Microbiol 2005;8:46-53.
84. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.
85. Drutz DJ. Fungal diseases. In: Sites DP, Terr AI, Parslow TG, editors. Basic and clinical immunology. East Norwalk: Appleton & Lange; 1994. p. 649-65.
86. Williams DL. Overview of (1, 3)-beta-D-glucan immunobiology. Mediators Inflamm 1997;6:247-50.
87. Netea MG, van der Graaf C, van der Meer JWM, Kullberg BJ. Recognition of fungal pathogens by Toll-like receptors. Eur J Clin Microbiol Infect Dis 2004;23:672-6.
88. Netea MG, Sutmuller R, Hermann C, van der Graaf CA, van der Meer JW, Adema G, et al. Toll-like receptor 2 inhibits cellular responses against *Candida albicans* through pathways mediated by IL-10 and CD4+CD25+ regulatory T cells. J Immunol 2004;172:3712-8.
89. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med 2003;197:1119-24.
90. Gantner BN, Simmons RM, Canavera SJ, Akirta S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 2003;197:1107-17.
91. Tokunaga T, Yamamoto S, Shimada S, Abe H, Fukuda T, Fujisawa Y, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984;72:955-62.
92. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, et al. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol 1992;36:983-97.
93. Bird AP. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acid Res 1980;8:1499-504.
94. Horner AA, Raz E. Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. J Allergy Clin Immunol 2002;110:706-12.
95. Hussain I, Kline JN. DNA, the immune system, and atopic disease. J Investig Dermatol Symp Proc 2004;9:23-8.
96. Wild JS, Sur S. CpG oligonucleotide modulation of allergic inflammation. Allergy 2001;56:365-76.
97. van Uden J, Raz E. Immunostimulatory DNA and applications to allergic disease. J Allergy Clin Immunol 1999;104:902-10.
98. Kitagi K, Jain VV, Businga TR, Hussain I, Kline JN. Immunomodulatory effects of CpG oligonucleotides on established Th2 responses. Clin Diagn Lab Immunol 2002;9:1260-9.
99. Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol 2000;165:4848-53.
100. Curotto de Lafaille MA, Muriglan S, Sunshine MJ, Lei Y, Kutchukhidze N, Furtado GC, et al. Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. J Exp Med 2001;194:1349-59.
101. Bohle B. CpG motifs as possible adjuvants for the treatment of allergic diseases. Int Arch Allergy Immunol 2002;129:198-203.
102. Roy SR, Schiltz AM, Marotta A, Shen Y, Liu AH. Bacterial DNA in house and farm barn dust. J Allergy Clin Immunol 2003;112:571-8.
103. Chen Y, Lenert P, Weerantna R, McCluskie M, Wu T, Davis HL, et al. Identification of methylated CpG motifs as inhibitors of the immune stimulatory CpG motifs. Gene Ther 2001;8:1023-32.
104. Pisetsky DS, Reich CF. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. Clin Immunol 2000;96:198-204.
105. Takabayashi K, Vibet L, Chisholm D, Zubeldia J, Horner AA. Intranasal immunotherapy is more effective than intradermal immunotherapy for the induction of airway allergen tolerance in Th2-sensitized mice. J Immunol 2003;170:3898-905.
106. Hall G, Houghton CG, Rahbek JU, Lamb JR, Jarman ER. Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production. Vaccine 2003;21:549-61.
107. Hawrylowicz CM, O'Garra AO. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 2005;5:271-83.
108. Akbari O, Stock P, DeKruyff RH, Umetsu DT. Mucosal tolerance and immunity: regulating the development of allergic disease and asthma. Int Arch Allergy Immunol 2003;130:108-18.
109. Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480-7.
110. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253-7.
111. Mills KHG, McGuirk P. Antigen-specific regulatory T cells—their induction and role in infection. Semin Immunol 2004;16:107-17.
112. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, et al. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 2002;8:625-9.
113. Curotto de Lafaille MA, Lafaille JJ. The role of regulatory T cells in allergy. Springer Semin Immunopathol 2004;25:295-310.
114. Lauener RP, Birchier T, Adamski J, Braun-Fahrländer C, Bufe A, Herz U, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet 2002;360:465.
115. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrländer C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004;113:482-8.

116. Raby BA, Klimecki WT, Laprise C, Renaud Y, Faith J, Lemire M, et al. Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. Am J Respir Crit Care Med 2002;166:1449-56.
117. Fageras Bottcher M, Hmani-Aifa M, Lindström A, Jenmalm MC, Mai XM, Nilsson L, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-linked interleukin-12(p70) responses in Swedish children. J Allergy Clin Immunol 2004;114:561-7.
118. Re F, Strominger JL. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. J Immunol 2004;173:7548-55.
119. McGuirk P, Mills KHG. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol 2002;23:450-5.

Clinical and E perimental Allergy, 2000, Volume 30, pages 1230–1234

Exposure to endotoxin or other bacterial components might protect against the development of atopy

E. VON MUTIUS*, C. BRAUN-FAHRLÄNDER , R. SCHIERL , J. RIEDLER , S. EHLERMANN , S. MAISCH*, M. WASER and D. NOWAK

*University Children s Hospital Munich, ermany, Institute of Social and Preventive Medicine, University of Basel, Switzerland, Institute for ccupational and Environmental Medicine, University Munich, ermany, Children s Hospital Salzburg, Austria

Abstract

Background Several recent studies have shown that growing up on a farm confers significant protection against the development of atopy. These findings point particularly towards the importance of exposure to stable dust and farm animals. It has furthermore been reported that endotoxin, an intrinsic part of the outer membrane of gram negative bacteria, is abundant in environments where livestock and poultry is kept.

The *aim* of this study was therefore to measure the level of environmental endotoxin exposure in homes of farmers' children, children with regular contact to livestock and control children with no contact to farm animals.

Methods Eighty-four farming and nonfarming families were identified in rural areas in Southern Germany and Switzerland. Samples of settled and airborne dust were collected in stables, and of settled dust indoors from kitchen floors and the children's mattresses. Endotoxin concentrations were determined by a kinetic Limulus assay.

Results Endotoxin concentrations were highest in stables of farming families, but were also significantly higher indoors in dust from kitchen floors (143 EU/mg vs 39 EU/mg, $P < 0.001$) and children's mattresses (49479 EU/m^2 vs 9383 EU/m^2, $P < 0.001$) as compared to control children from nonfarming families. In addition, endotoxin levels were also significantly higher in mattresses and dust from kitchen floors in households where children had regular contact to farm animals (38.6 EU/mg and 23340 EU/m^2, respectively) as compared to control subjects.

Conclusion We propose that the level of environmental exposure to endotoxin and other bacterial wall components is an important protective determinant for the development of atopic diseases in childhood.

Keywords: atopy, endotoxin, children

Clinical and E perimental Allergy, Vol. 30, pp. 1230–1234. Submitted 15 November 1999; revised 2 February 2000; accepted 24 May 2000.

Introduction

Growing up on a farm confers significant protection against the development of atopy as several recent reports have consistently shown 1–4 . In a Swiss population ($n = 1620$) of 6–15-year-old schoolchildren the odds of having seasonal symptoms of hay fever (adj.OR = 0.34, 95% CI: 0.12–0.89) and of developing atopic sensitization as measured by RAST (adj.OR = 0.31, 95% CI: 0.13–0.73) were strongly decreased in children raised on a farm as compared to their peers from the same rural area whose parents were not farmers 1 . Similarly, in a large survey of Bavarian children entering school at age 5–7 years ($n = 10\,163$) the prevalence of hay fever among children raised on a farm was significantly lower than among their peers from the same villages who did not grow up on a farm (1.8% vs 4.9%, $P \leq 0.001$) 2 . Adjustment for potential

Correspondence: Dr E.von Mutius, Klinikum Innenstadt, Lindwurmstr.4, D-80337 München, Germany.

confounding variables confirmed the strong inverse relation. A recent Austrian survey of 8–10-year-old children living in a rural area has furthermore confirmed the findings. Farmers' children were at significantly lower risk to develop hay fever (3.1% vs 10.3%, $P = 0.0002$) and atopic sensitization to a panel of aeroallergens (adj.OR = 0.48, 95% CI: 0.30–0.75) than children whose parents did not run a farm [3]. In all three surveys a slight protective effect was also seen for the prevalence of asthma (e.g. adj.OR = 0.65, 95% CI: 0.39–1.09 in Bavaria), whereas the development of atopic eczema was not affected by farming activities of the parents.

Living conditions of farming families differ in many respects from life styles of other families: larger family size, more pets, frequently heating with wood or coal, less maternal smoking, more dampness, and other dietary habits [1]. None of these factors could however, explain the strong inverse association between atopy and growing up on a farm. In contrast, contact to livestock and poultry was found to explain much of the relation between farming and atopy in the Austrian study [3]. Likewise, in the Bavarian survey a strong inverse, dose-dependent relation between exposure to livestock and the prevalence of atopic diseases was found (adj.OR = 0.41, 95% CI: 0.23–0.74 for frequent contact to livestock) [2]. Furthermore, Austrian children with regular contact to farm animals but not growing up on a farm had a significantly lower prevalence of atopic sensitization (13.5% vs 34.8%, $P = 0.01$) than children with no contact [3]. These findings suggest that factors prevalent in stables and presumably thereby also in the homes of farming families confer the protection which is associated with a farming life style.

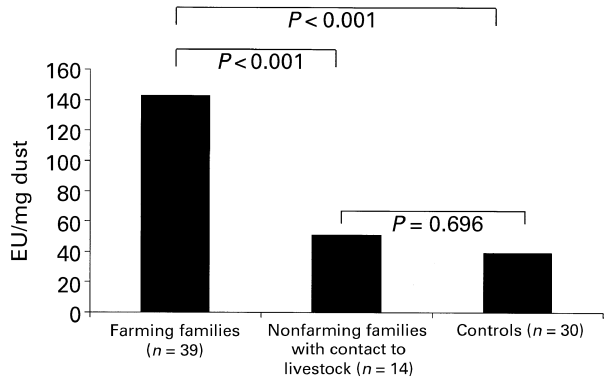
Previous reports have indicated that several exposures are particularly high in stables such as exposure to moulds, ammonia, faeces, animal proteins, constituents of feed and endotoxin. Endotoxin is an intrinsic part of the outer membrane of gram negative bacteria, thereby activating TH1-type immune responses and thus interfering with the development of TH2-type immune responses resulting in atopy. We therefore aimed to measure concentrations of endotoxin not only in dust samples of Bavarian and Swiss stables, but also indoors in the homes of farmers' and nonfarmers' children with contact to farm animals. We furthermore wished to compare these levels to endotoxin concentrations in homes of children without regular contact to livestock or poultry.

Methods

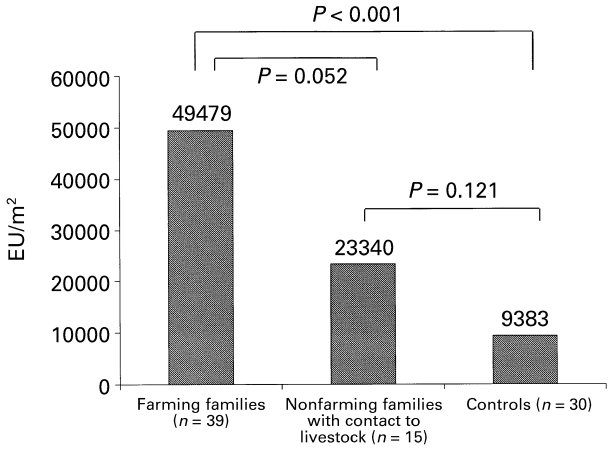
Eighty-four families with 146 children aged 1–14 years ($n = 119$ in Bavaria and $n = 27$ in Switzerland) were enrolled in the study through personal contacts, advertisements in agricultural news letters and as part of the ongoing environment and health monitoring program SCARPOL in Switzerland [5]. There, the parents of all 14-year-old children of the village of Grabs ($n = 97$) visiting the school physician were asked to complete a questionnaire and to consent to dust sampling. Ninety-one families completed the questionnaire and 63 (65%) accepted dust collection. Of those were 10 children living on a farm and eight nonfarming children with regular contact to farm animals. All farms in Switzerland and Bavaria were run by the family, all kept cattle and some families additionally kept a few pigs, sheep, goats or horses. All families in Bavaria and Switzerland were living in rural areas, in total 39 families lived on a farm where livestock was kept, and 45 families were not living on a farm. Of these 45 families, subjects in 15 families had regular contact to livestock through their neighbours. Exposure to livestock in nonfarmers children was defined prior to endotoxin analysis and assumed if parents reported that the child had regular (at least once a week) contact to either cattle, pigs, sheep, poultry, horses or goats.

Settled dust was taken in the stables of farming families at several locations where children were usually playing. In addition, 30 airborne dust samples were collected in each of 30 stables on 37 mm diameter glass fibre filters (SKC Müllheim, Germany) housed in respirable ($PM_{2.5} \leq 2.5\ \mu m$) and inhalable ($PM_{7.5} \leq 7.5\ \mu m$) dust samplers (Personal air sampler, GSA Me gerätebau Neuss, Germany) with suction provided by battery-operated pumps (Alpha-1, air sampler, Ametek, USA) operated at 3.5 L/min (inhalable fraction) or 2.0 L/min (respirable fraction). The mean sampling duration was 3.1 h. Endotoxin concentrations were expressed as EU/m^3 air. Furthermore, dust samples from kitchen floors and children's mattresses were taken using a vacuum cleaner of 1200 W operated for 2 min at full power. ALK filters were used for dust collection according to the ISAAC Phase II manual [6]. Careful attention was given to ensure comparability of the method of dust collection at the different sites. In one family only dust samples from mattresses, but not from kitchen floors were taken. All samples were stored at room temperature [7] and analysed within 1 week after collection.

Endotoxin content of all dust samples was determined by a kinetic Limulus assay as described by Hollander and coworkers [8] in the laboratory of the Institute for Occupational and Environmental Medicine of the University of Munich. Briefly, each filter was extracted by rapid shaking with endotoxin-free water (7.5 mL) for one hour. From a diluted (air sample 1 : 10, dust samples 1 : 100 till 1 : 10000) aliquot, 100 μL were added to a microtitre-plate well (96 wells, NUNC) and assayed with LAL reagent (kinetic- CL, BioWhittaker) at 37.0 °C. A standard calibration curve (50, 5, 0.5, 0.005 EU/mL) was performed on each plate. Optical density at 405 nm was measured by an automatic reader (PowerWave™, MWG Biotech). The intra-assay variability was less than 5%, whereas the interassay variability was



ig. 1. Levels of endotoxin in kitchens (in EU/mg dust) of farmers children, children with regular contact with livestock, and control children.



ig. 2. Levels of endotoxin in mattresses (in EU/m^2) of farmers children, children with regular contact with livestock, and control children.

lower than 20%. For dust samples collected from kitchen floors, results were related to the dust quantity and expressed as EU/mg. Results of dust samples from mattresses were related to the size of the sampled mattress and expressed as EU/m^2.

Because of non-normal distribution of exposure levels the geometric mean (gm) with 95% confidence (95% CI) intervals of dust and endotoxin exposures were calculated. Comparisons between groups were computed using the *t*-test of log transformed values. *P*-values were adjusted by the Bonferroni procedure to account for multiple testing.

The study was performed from December 1998 to June 1999.

Results

Samples of settled dust were collected from 38 stables of farming families ($n = 30$ in Bavaria and $n = 8$ in Switzerland). In addition, dust samples of kitchen floors of 84 farming and nonfarming households ($n = 57$ in Bavaria and $n = 27$ in Switzerland) and dust samples of children's mattresses of 83 farming and nonfarming families ($n = 56$ in Bavaria and $n = 27$ in Switzerland) were collected. As several samples within one family were taken, averages of exposure per household were calculated and compared between groups. As one might expect endotoxin exposures were highest in the stables (gm = 649 EU/mg, 95% CI = 503–838 EU/mg). Interestingly, concentrations of endotoxin were significantly ($P = 0.0001$) higher in the inhalable fraction (gm = 150 EU/m^3, 95% CI = 83–272 EU/m^3) than in the respirable fraction (gm = 7 EU/m^3, 95% CI = 4–12 EU/m^3).

More dust was collected from kitchen floors of farming families (gm = 797 mg, 95% CI = 670–949 mg) than in both families of children with regular contact to livestock (gm = 449 mg, 95% CI = 292–692 mg) and control households (gm = 434 mg, 95% CI = 342–552 mg) ($P = 0.011$ and $P < 0.001$, respectively). The endotoxin exposures per mg dust were significantly higher in dust samples from kitchen floors of farming families (gm = 143 EU/mg, 95% CI = 114–180 EU/mg) as compared to nonfarming families (children with livestock contact: gm = 51 EU/mg, 95% CI = 30–89 EU/mg; controls: gm = 39 EU/mg, 95% CI = 30–50 EU/mg) (Fig. 1). Levels of endotoxin were also significantly higher in mattresses of farming families (gm = 49479 EU/m^2, 95% CI = 34951–70047 EU/m^2) and marginally higher in families with children having regular contact to livestock (gm = 23340 EU/m^2, 95% CI = 11159–48819 EU/m^2) as compared to the control group (gm = 9383 EU/m^2, 95% CI = 5882–14968 EU/m^2) (Fig. 2). When expressing endotoxin levels in mattresses as EU/mg dust or when comparing differences in exposure on a personal rather than household level similar results were obtained (data not shown).

Discussion

Endotoxin concentrations were higher in homes of children living on a farm as compared to homes of nonfarming families. Furthermore, in families of children with regular contact to livestock higher endotoxin concentrations were found in the mattress as compared to control households. These findings suggest that contact to livestock not only strongly increases LPS exposure while staying in a stable, but also augments daily exposure indoors.

Endotoxin consists of a family of molecules called lipopolysaccharides (LPS) and is an intrinsic part of the outer membrane of gram negative bacteria 9 . LPS and other bacterial wall components which can also be found abundantly in stables where pigs, cattle and poultry is kept

10,11 , engage with antigen-presenting cells via CD14 ligation eliciting strong IL-12 responses 12 . IL-12, in turn, is regarded as an obligatory signal for the maturation of naive T cells into Th1-type cells 13 . There is ample evidence that IgE responses are down regulated by inhibitory signals derived from Th1-type cells 14 . Recent studies have furthermore shown that a polymorphism in the 5′-flanking region of the CD14 gene on chromosome 5q31.1 results in down-regulation of IgE levels 15 lending further support to the hypothesis that (via CD14 ligation) LPS and other bacterial wall components affect the levels of serum IgE and thus interfere with the development of atopy.

Bacterial signals may, however, only play a role in promoting T helper cell differentiation and polarization at the time of development of primary immune responses 16 . There is increasing evidence suggesting that the predominant type of response (Th1-like or Th2-like) to a given antigen is determined at the time of the primary encounter with the antigen 17 , a life period during which the Th2-polarization characteristic of the fetal immune system is being progressively replaced by Th1-dominance 18,19 . The pivotal role of the antigen presenting cell to induce differentiation of Th1-type effector and memory cells early in life is increasingly recognized 12 . These cells are generated in the bone marrow, but migrate to peripheral tissues, including the airway epithelium, and to local lymph nodes. LPS and other bacterial wall components bound to particles of various sizes are likely to penetrate in various depths into the airways and to exert their immune stimulating effects on cells of the upper and lower airway tract. The fact that a higher proportion of endotoxin was bound to larger particle size in our samples as in previous studies 20 may relate to the preferential effect on the reduction in the prevalence of hay fever as compared to asthma in farmers' children. In adult life, endotoxin exposure has been shown to induce strong inflammatory responses via production of IL-1 IL-6, IL-8, and TNFα resulting in neutrophilia and fever 21 , but also to induce airway hyper-responsiveness and respiratory symptoms 22,23 .

Endotoxin is ubiquitous in nature, being present in normal indoor environments as a constituent of house dust 24 . It has previously been suggested that endotoxin levels increase where animals are kept indoors, and it seems noteworthy that several recent studies have shown a protective effect of early exposure to cats and dogs on the development of atopic sensitization 25,26 . Furthermore, endotoxin levels are likely to be particularly high in developing countries where poultry and livestock are kept in close proximity to human housing. In this context, it is of interest to note that a previous study from Africa has found a significantly decreased odds ratio for the development of atopic senitization (aOR = 0.49, 95% CI: 0.28–0.87) in children in whose homes pigs were kept 27 .

The limitations of exposure studies as the one presented here are well-known. In particular, although we know that endotoxin exposure is associated with farming and exposure to livestock, and that farming is associated with a decreased risk of atopy, we do not know whether this applies to individuals within these communities. High exposure levels to endotoxin may be a surrogate for other aspects of exposure to stables which are associated with a decreased risk of atopy. It seems, however, unlikely that exposure to moulds which are also abundant in stables confers protection for the development of atopy, since most studies investigating the effects of exposure to moulds or dampness have either reported no effect or an increased risk to develop childhood wheeze and asthma 28 . Other bacterial wall components from gram positive bacteria or atypical mycobacteria are likely to affect immune responses in similar ways as LPS 29,30 . Particularly, listeria, a germ also abundant in stables, is known to induce IL-12 production 31 thereby potentially deviating the immune response towards a Th1-like phenotype. Recent studies furthermore suggest that certain motifs of bacterial DNA might also prevent the development of atopy 32 .

We therefore propose that the level of environmental exposure to endotoxin and other bacterial wall components is an important protective determinant for the development of atopic diseases in childhood. Further understanding of these mechanisms may in the future open a novel avenue for the prevention of atopic diseases in childhood.

Acknowledgements

This study was supported by the Bavarian State Ministry for Environmental Protection.

References

1 Braun-Fahrländer Ch, Gassner M, Grize L, et al. Prevalence of hay fever and allergic sensitization in farmers's children and their peers living in the same rural community. Clin Exp Allergy 1999; 29:28–34.
2 von Ehrenstein O, von Mutius E, Illi S, Baumann L, Böhm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy 2000; 30:187–93.
3 Riedler J, Eder W, Oberfeld G, Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitisation. Clin Exp Allergy 2000; 30:194–200.
4 Aberg N. Asthma and allergic rhinitis in Swedish conscripts. Clin Exp Allergy 1989; 19:59–63.
5 Braun-Fahrländer Ch, Gassner M, Grize L, et al. Respiratory health and long term exposure to air pollutants in Swiss school children. Am J Respir Crit Care Med 1997; 155:1042–9.
6 ISAAC Steering Committee. Phase II modules of the International Study of Asthma and Allergies in Childhood (ISAAC). Münster, 1998.

7 Milton DK, Johnson DK, Park JH. Environmental endotoxin measurement: interference and sources of variation in the Limulus assay of house dust. Am Ind Hyg Assoc J 1997; 58:861–7.

8 Hollander A, Heederik D, Verslot P, Douwes J. Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. Am Ind Hyg Assoc J 1993; 54:647–53.

9 Rietschel ET, Schade U, Jensen M, Wollenweber HW, Lüderitz O, Greisman SG. Bacterial endotoxins: chemical structure, biological activity, and role in septicaemia. Scand J Infect Di Suppl 1982; 31:8–21.

10 Thedell TD, Mull JC, Olenchock SA. A brief report of gram-negative bacterial endotoxin levels in airborne and settled dust in animal confinement buildings. Am J Indust Med 1980; 1:3–7.

11 Dutkiewicz J. Exposure to dust-borne bacteria in agriculture. I. Environmental studies. Arch Environ Health 1978; 33:250–9.

12 Martinez FD. Maturation of immune responses at the beginning of asthma. J Allergy Clin Immunol 1999; 103:355–61.

13 Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol 1995; 154:5071–9.

14 Maggi E, Parronchi P, Manetti R, Simonelli C, Piccinni F, Rugiu FS, De Carli M, Ricci M, Romagnani S. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human TH1 and TH2 clones. J Immunol 1992; 148:2142–7.

15 Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A polymorphism in the 5′-flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum IgE. Am J Respir Cell Mol Biol 1999; 20:976–83.

16 Holt PG, Sly PD, Björkstén B. Atopic versus infectious diseases in childhood: a question of balance? Pediatr Allergy Immunol 1997; 8:53–8.

17 Holt PG. Primary allergic sensitization to environmental antigens: perinatal T-cell priming as a determinant of responder phenotyype in adulthood. J Exp Med 1996; 183:1297–301.

18 Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. Lancet 1999; 353:196–200.

19 Lee SM, Suen Y, Chang L, Bruner V, uian J, Indes J, Knoppel E, van de Ven C, Cairo MS. Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of interferon-γ, natural killer, and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells. Blood 1996; 88:945–54.

20 Vinzents P, Nielsen BH. Variations in exposures to dust and endotoxin in Danish piggeries. Am Hyg Assoc J 1992; 53:237–41.

21 Jagielo PJ, Thorne PS, Watt JL, Frees KL, uinn TJ, Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. Chest 1996; 110:263–70.

22 Michel O, Duchateau J, Sergysels R. Effects of inhaled endotoxin on bronchial reactivity in asthmatics and normal subjects. J Appl Physiol 1989; 66:1059–64.

23 Heederik D, Brouwer R, Biersteker K, Boleij JSM. Relationship of airborne endotoxin and bacteria levels in pig farms with the lung function and respiratory symptoms of farmers. Int Arch Occup Environ Health 1991; 62:595–601.

24 Michel O, Ginanni R, Duchateau J, Vertongen F, Le Bon B, Sergysels R. Domestic endotoxin exposure and clinical severity of asthma. Clin Exp Allergy 1991; 21:441–8.

25 Hesselmar B, Aberg N, Aberg B, Eriksson B, Björkstén B. Does early exposure to cat or dog protect against later allergy development? Clin Exp Allergy 1999; 29:611–7.

26 Svanes C, Jarvis D, Chinn S, Burney P. Childhood environment and adult atopy: results from the European Community Respiratory Health Survey. J Allergy Clin Immunol 1999; 103:415–20.

27 Shaheen SO, Aaby P, Hall AJ, Barker DJP, Heyes CB, Shiell AW, Goudiaby A. Measles and atopy in Guinea-Bissau. Lancet 1996; 347:1792–6.

28 Nicolai T, Illi S, von Mutius E. Effect of dampness at home in childhood on bronchial hyperreactivity in adolescence. Thorax 1998; 53:1035–40.

29 Abou-Zeid C, Gares MP, Inwald J, Janssen R, Zahng Y, Young D, et al. Induction of a type 1 immune responses to a recombinant antigen from Mycobacterium tuberculosis expressed in Mycobacterium vaccae. Infect Immun 1997; 65:1856–62.

30 Cleveland MG, Gorham JD, Murphy TL, Toumanen E, Murphy KM. Lipoteichooic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 1996; 64:1906–12.

31 Yeung VP, Gieni RS, Umetsu DT, DeKruyff RH. Heat-Killed Listeria monocytogenes as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. J Immunol 1998; 161:4146–52.

32 Jahn-Schmid B, Wiedermann U, Bohle B, Repa A, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs modulate the allergic Th2 response of BALB/c mice to Bet v1, the major birch pollen allergen. J Allergy Clin Immunol 1999; 104:1015–23.

Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells

CLAUDIA ZUANY-AMORIM[1], ELZBIETA SAWICKA[1,2], CORINNE MANLIUS[1], ALAIN LE MOINE[3], LAURA R. BRUNET[4], DAVID M. KEMENY[2], GARETH BOWEN[5], GRAHAM ROOK[4] & CHRISTOPH WALKER[1]

[1]*Novartis Horsham Research Centre, Novartis Pharmaceutical Ltd. Wimblehurst Road, Horsham, UK*
[2]*King's College London, Rayne Institute, London, UK*
[3]*Laboratory of Experimental Immunology, Université Libre de Bruxelles, Belgium*
[4]*Department of Medical Microbiology, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, London, UK*
[5]*SR Pharma, Centre Point, London, UK*
Correspondence should be addressed to C.Z.-A.; email: claudia.zuany-amorim_fromond@pharma.novartis.com

Allergic asthma is a chronic inflammatory disease and despite the introduction of potent and effective drugs, the prevalence has increased substantially over the past few decades[1]. The explanation that has attracted the most attention is the 'hygiene hypothesis', which suggests that the increase in allergic diseases is caused by a cleaner environment and fewer childhood infections[2–4]. Indeed, certain mycobacterial strains can cause a shift from T-helper cell 2 (Th2) to Th1 immune responses, which may subsequently prevent the development of allergy in mice[5–7]. Although the reconstitution of the balance between Th1 and Th2 is an attractive theory, it is unlikely to explain the whole story, as autoimmune diseases characterized by Th1 responses can also benefit from treatment with mycobacteria and their prevalence has also increased in parallel to allergies[8]. Here we show that treatment of mice with SRP299, a killed *Mycobacterium vaccae*-suspension, gives rise to allergen-specific $CD4^+CD45RB^{Lo}$ regulatory T cells, which confer protection against airway inflammation. This specific inhibition was mediated through interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), as antibodies against IL-10 and TGF-β completely reversed the inhibitory effect of $CD4^+CD45RB^{Lo}$ T cells. Thus, regulatory T cells generated by mycobacteria treatment may have an essential role in restoring the balance of the immune system to prevent and treat allergic diseases.

We analyzed the effect of SRP299 treatment on the ovalbumin (OVA)-induced eosinophilic airway inflammation using two different immunization protocols. A single OVA aerosol challenge resulted in a significant increase in the number of eosinophils, neutrophils, lymphocytes and macrophages in bronchoalveolar lavage (BAL) fluid from both the short-term (21 d, two immunizations) as well as the long-term (63 d, four immunizations) protocols (Fig. 1*a*). Subcutaneous (s.c.) injection of 0.1 mg SRP299 (optimal dose; ref. 9 and data not shown) three weeks before the first OVA immunization significantly reduced the airway eosinophilia using both immunization protocols (Fig. 1*a*). The inhibitory effect of SRP299 treatment on the allergic inflammation was not restricted to eosinophils but also affected other inflammatory cell types (Fig. 1*a*).

Cultures of OVA-stimulated splenocytes from saline-treated and OVA-immunized mice (short-term protocol) contained substantial amounts of interleukin-4 (IL-4), IL-5 and IL-10 (Fig. 1*b*), whereas the antigen stimulation failed to trigger significant production of interferon-γ (IFN-γ), IL-2 and IL-12. Cultures of splenocytes from immunized and SRP299-treated mice contained significantly higher levels of IL-5 and IL-10, whereas the production of IL-4 was not altered. No significant differences were found in IFN-γ, IL-2 and IL-12 levels, irrespective of treatment, suggesting that the mechanisms by which SRP299 induces the suppression of the allergic inflammatory response is not due to a diversion from allergen-specific T-helper cells (Th2) to Th1 cells.

The increased production of IL-5 and IL-10 in SRP299-treated mice without corresponding increase in Th1-associated cytokines may indicate that SRP299 induces the generation of IL-10-producing regulatory cells that might specifically block the allergic response over a long period of time[10–12]. We therefore investigated whether splenocytes from SRP299-treated mice could inhibit airway eosinophilia in immunized recipient mice. Intravenous transfer of 2×10^6 splenocytes from OVA-primed SRP299-treated mice to OVA-immunized recipient mice, 24 h before the antigen challenge, significantly reduced OVA-induced airway eosinophilia (Fig. 2*a*). A less pronounced inhibitory effect was observed in recipient mice injected with splenocytes from SRP299-treated and PBS-challenged mice. By contrast, the transfer of splenocytes from saline-treated and PBS- or OVA-primed as well as from naive mice into OVA-immunized mice failed to inhibit the antigen-induced airway eosinophilia.

We next sought to identify the cell population responsible for the inhibition of the allergic response. We purified T cells from the spleen of OVA-primed SRP299-treated mice and tested their regulatory activity in OVA-immunized recipient mice. Only treatment of mice with 2×10^6 purified T cells significantly reduced the antigen-induced airway eosinophilia (Fig. 2*a*). Furthermore, only $CD4^+$ and not $CD8^+$ T cells were able to do so. The regulatory activity of the $CD4^+$ T cells from SRP299-treated mice is highly potent as cell numbers as low as 0.25×10^6 significantly suppressed allergen-induced eosinophilic inflammation (Fig. 2*b*). The BAL fluid of mice immunized and challenged following the transfer of purified $CD4^+$ T cells from SRP299-treated mice contained significantly increased levels of IL-10, whereas no significant changes in IL-4 or transforming growth factor-β (TGF-β) were observed (Fig. 2*c*). In addition, transfer of purified $CD4^+$ splenocytes from OVA-primed SRP299-treated mice to OVA-immunized and challenged recipient mice significantly re-

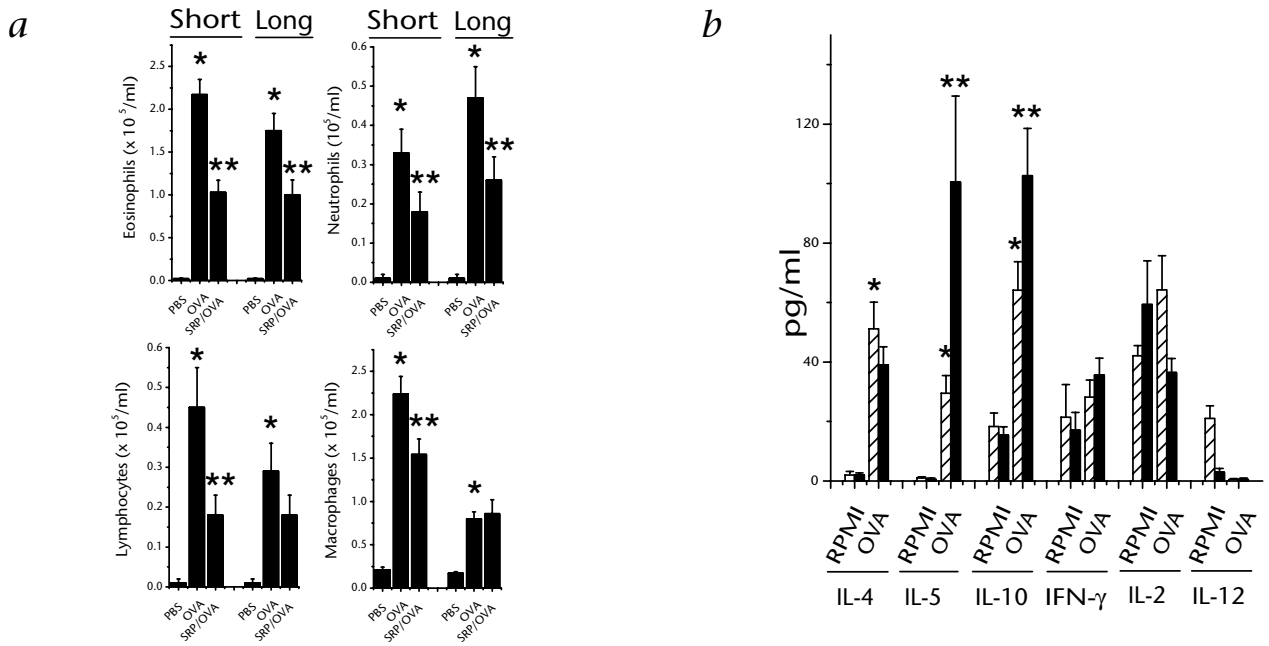


Fig. 1 Effect of SRP299 treatment on the OVA-induced eosinophilic inflammation and cytokine production by splenocytes. ***a***, Effect of SRP299 on OVA-induced changes in the cell numbers in mice immunized using a short-term (21 d, two immunizations) and long-term (63 d, four immunizations) protocols. OVA-immunized mice were treated with saline and challenged by aerosol with either PBS (PBS) or OVA (OVA) or treated s.c. with SRP299 21 d before the beginning of immunization and challenged with OVA (SRP/OVA). 48 h after the aerosol PBS or OVA challenge, BAL fluid was collected and cell numbers determined. Data are means ± s.e.m.; n = 7–10 per group. *, $P < 0.05$; **, $P < 0.05$ versus saline-treated and PBS- or OVA-challenged mice, respectively. ***b***, Effect of SRP299 on IL-2, IL-4, IL-5, IL-10, IL-12 and IFN-γ release in response to *in vitro* antigen-mediated activation of splenocytes from OVA-immunized mice. Mice were treated s.c. 21 d before the OVA immunization either with saline (▨) or with SRP299 (■) and killed on day 21 of immunization. Splenocytes were incubated with RPMI alone or were stimulated with OVA and the cytokine levels in the cell-free supernatants were determined by ELISA. Data are means ± s.e.m. of 5–7 mice per group. **, $P < 0.05$; *, $P < 0.05$ versus saline-treated and RPMI- or OVA-stimulated cells, respectively.

duced subsequent allergen induced bronchial hyperresponsiveness (BHR) to methacholine (Fig. 2*d*). These data clearly demonstrate that SRP299-induced regulatory T cells not only control the extent of the allergen-induced eosinophilic inflammation, but also affect more pathophysiological readouts such as BHR.

In an attempt to verify the specificity of the $CD4^+$ regulatory T cells, we treated cockroach extract antigen (CEA)-immunized mice with $CD4^+$ T cells generated in mice treated with SRP299 and immunized and challenged with OVA or CEA. Pretreatment with $CD4^+$ T cells from SRP299-treated and CEA-immunized, but not from OVA-immunized mice inhibited the CEA-induced eosinophilic inflammation in CEA-immunized recipient mice (Fig. 2*e*), demonstrating an allergen-specific effector function of these regulatory T cells. This conclusion is further supported by the observation that the transfer of $CD4^+$ T cells from SRP299-treated and OVA-immunized mice into CEA-immunized mice significantly inhibited the allergen-induced eosinophilic inflammation when challenged with both CEA and OVA.

Several studies have demonstrated that the regulatory T cells are predominantly found within the $CD4^+CD45RB^{Lo}CD25^+$ subpopulation[13]. The flow-cytometric analysis of the distribution of CD45RB and CD25 expressing $CD4^+$ T cells obtained from spleens revealed no significant differences between SRP299-treated or saline-treated, OVA-immunized mice (data not shown). However, the transfer of FACS-sorted $CD4^+$ T cells expressing low levels of CD45RB ($CD45RB^{Lo}$) from SRP299-treated, OVA-immunized mice into recipient OVA-immunized mice before challenge significantly reduced the allergen-induced eosinophilic inflammation (Fig. 3*a*). In contrast, the transfer of high-level CD45RB-expressing $CD4^+$ T cells ($CD45RB^{Hi}$) had no suppressive effect, nor did injection of either CD45RB populations from saline-treated mice.

A characteristic feature of regulatory T cells is their ability to inhibit the proliferation of other T cells *in vitro*[14,15]. Fig. 3*b* shows that $CD4^+CD45RB^{Lo}$ T cells from SRP299-treated and OVA-immunized mice added to cultures of $CD4^+$ T cells from untreated OVA-immunized mice significantly inhibited the antigen-induced proliferative response *in vitro*. The suppression of the proliferative response of OVA-specific T cells induced by $CD4^+CD45RB^{Lo}$ T cells from SRP299-treated mice was completely reversed by adding neutralizing antibodies against IL-10 and TGF-β.

In an attempt to quantify the number of SRP299-induced regulatory T cells, $CD4^+$ T cells from both SRP299- or saline-treated OVA-immunized mice were stimulated *in vitro* with the allergen and the number of IL-10 producing cells were analyzed within the different CD45RB populations using flow cytometry. Fig. 3*c* shows a representative dot plot of membrane CD45RB and intracellular IL-10 double-stained $CD4^+$ T cells, demonstrating that the stimulation of these cells with OVA induces the production of IL-10 in both, $CD45RB^{Lo}$ and $CD45RB^{Hi}$ cells. The quantitative analysis revealed a significantly increased number of IL-10-producing $CD4^+$ cells in cultures from SRP299-treated compared with saline-treated mice (data not shown), which was mainly due to an increase in IL-10-producing $CD45RB^{Lo}$ cells (Fig. 3*d*). Qualitatively similar changes were found measuring the IL-10 levels in supernatants of these cultures (Fig. 3*e*).

Next, we examined the potential role of IL-10 and TGF-β in the regulation of the *in vivo* inhibitory activity. Pre-treatment of

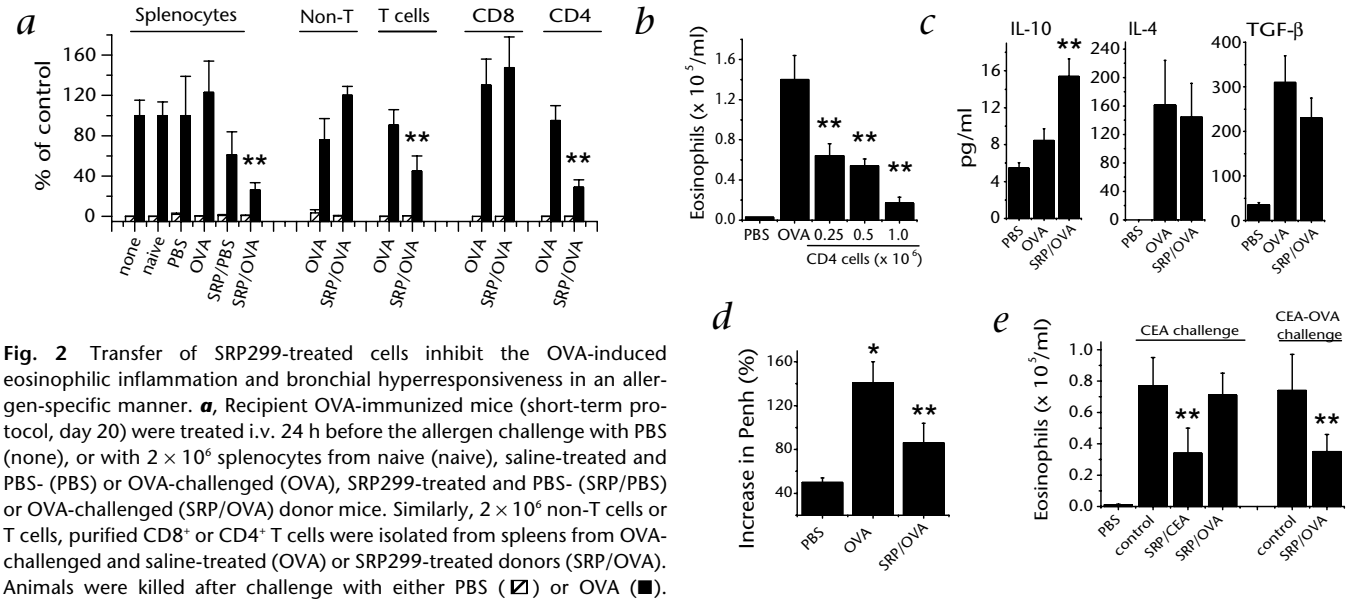


Fig. 2 Transfer of SRP299-treated cells inhibit the OVA-induced eosinophilic inflammation and bronchial hyperresponsiveness in an allergen-specific manner. ***a***, Recipient OVA-immunized mice (short-term protocol, day 20) were treated i.v. 24 h before the allergen challenge with PBS (none), or with 2×10^6 splenocytes from naive (naive), saline-treated and PBS- (PBS) or OVA-challenged (OVA), SRP299-treated and PBS- (SRP/PBS) or OVA-challenged (SRP/OVA) donor mice. Similarly, 2×10^6 non-T cells or T cells, purified $CD8^+$ or $CD4^+$ T cells were isolated from spleens from OVA-challenged and saline-treated (OVA) or SRP299-treated donors (SRP/OVA). Animals were killed after challenge with either PBS (▨) or OVA (■). Results are % of OVA-challenged control mice without cell transfer. Data are means ± s.e.m.; $n = 5$–8 per group. **, $P < 0.05$ versus control OVA-challenged mice (none). ***b***, Dose response data of purified $CD4^+$ T cells obtained from SRP299-treated and OVA-immunized and challenged mice, adoptively transferred into OVA-immunized recipient mice. Results are number of eosinophils present in BAL fluid obtained 48 h after the challenge with either PBS (PBS) or OVA (all other bars). Data are means ± s.e.m.; $n = 5$–8 per group. **, $P < 0.05$ versus control OVA-challenged mice (OVA). ***c***, BAL fluid IL-10, IL-4 and TGF-β levels in OVA-immunized mice challenged with PBS (PBS) or OVA (OVA) following transfer of no cells or purified $CD4^+$ T cells (1×10^6 cells/ml) from SRP299 treated mice as described above (SRP/OVA). Data are means ± s.e.m.; $n = 5$–8 per group. **, $P < 0.05$ versus control OVA-challenged mice (OVA). ***d***, Antigen-induced bronchial hyperreactivity to inhaled methacholine measured as increase in Penh from PBS- (PBS) or OVA-challenged mice (OVA) following transfer of none or purified $CD4^+$ T cells (1×10^6 cells/ml) from SRP299-treated mice as described above (SRP/OVA). Data are means ± s.e.m.; $n = 10$ per group. **, $P < 0.05$ versus control OVA-challenged mice. ***e***, Mice pre-immunized with CEA were i.v. treated with PBS (control) or with 1×10^6 $CD4^+$ T cells from SRP299-treated and OVA-immunized and challenged mice (SRP/OVA) or SRP299-treated and CEA-immunized and challenged mice (SRP/CEA). BAL was performed 48 h after the intranasal challenge with CEA alone (CEA challenge) or with OVA and CEA (CEA-OVA). For the latter, mice received an aerosol challenge of OVA followed by an intranasal challenge with CEA, 6 h later. Data are means ± s.e.m.; $n = 5$–7 per group. **, $P < 0.05$ versus PBS-treated and CEA-challenged mice.

recipient mice with neutralizing antibodies to either IL-10 or TGF-β significantly reduced the $CD4^+CD45RB^{Lo}$ T-cell–mediated suppressive effect, suggesting that both cytokines are required for the regulation of the eosinophilic inflammation by regulatory T cells (Fig. 3*f*). Indeed, treatment with antibodies against both IL-10 and TGF-β completely reversed the inhibition due to SRP299-induced OVA-specific $CD4^+CD45RB^{Lo}$ T cells. In contrast, neither the anti-cytokine nor the isotype-matched control antibodies had any effect on the OVA-induced inflammation in control mice. Taken together, these findings demonstrate that SRP299 treatment gives rise to regulatory $CD4^+CD45RB^{Lo}$ T cells, which display a specific protective effect on allergic pulmonary inflammation through IL-10 and TGF-β production.

Down-regulation of allergic responses by *mycobacteria in vivo* has been observed in numerous cases. However, the mechanism by which *mycobacteria* inhibit the inflammatory response remains unclear and various mechanisms, such as the induction of Th1 cytokines such as IFN-γ, have been proposed[5]. However, we were unable to demonstrate any significant changes in IFN-γ production following SRP299 treatment. In addition, the inhibitory effect was not confined to eosinophils but also affected all other cell types, suggesting that mechanisms other than simple changes in the balance between Th1 and Th2 must be responsible for the observed effects.

It is now well established that regulatory T cells can inhibit harmful immunopathological responses, but many key aspects, such as their antigen specificities, the cellular and molecular pathways involved in their development and mechanism of action remain unresolved. Different regulatory T-cell populations have been described in both the murine and human systems[13]. For example, regulatory T cells induced *in vitro* produce high levels of IL-10 but not IL-4, do not proliferate after stimulation and inhibit antigen-specific proliferation and cytokine production in a cell contact–dependent manner[16]. In addition, IL-10-producing regulatory T cells were shown to actively modulate a Th2-type response and immunoglobulin E (IgE) production[17]. Moreover, the protective mechanism by which $CD4^+CD45RB^{Lo}$ regulatory T cells inhibit the development of inflammatory bowel disease or prevent graft rejections in adoptive transfer experiments is critically dependent on the production of IL-10 and TGF-β (refs. 18,19). The regulatory T-cell described here is characterized as $CD4^+CD45RB^{Lo}$ T-cell subset producing and/or inducing the production of IL-10 and TGF-β. It is capable of inhibiting antigen-mediated proliferative responses and specifically suppresses allergen-induced eosinophilic inflammation. The phenotype and cytokine profile is therefore in line with the regulatory T cells described elsewhere[13]. Furthermore, treatment with SRP299 gives rise to the development of newly generated, allergen-specific regulatory T cells. This was clearly shown in the experiments where the adoptive transfer of purified $CD4^+$ T cells from SRP299-treated, OVA-immunized mice into CEA-immunized and challenged mice had no effect on the eosinophilic inflammation, whereas the double challenge with both CEA and OVA induced a significant inhibition. The IL-10 and TGF-β dependency of the observed inhibition was clearly demonstrated using neutralizing antibodies. In contrast, however, only small

increases in IL-10, but not TGF-β were found in BAL fluid or cell cultures from SRP299-treated mice. It is reasonable to assume that data generated with neutralizing antibodies best reflect the underlying mechanisms as cytokine levels in supernatants and diluted BAL fluid do not account for cell–cell interactions, temporal relationships between production and utilization or responses specific to the target organ.

In conclusion, regulatory CD4+ T cells generated by SRP299 treatment may have an essential role in restoring the balance of the immune system to prevent allergic diseases and offer advantages over treatments aimed at inducing a shift from an allergen-specific Th2 to Th1 response. Preliminary clinical studies with *M. vaccae* treatment in adults with asthma and rhinitis or in children with atopic dermatitis demonstrated clinical benefits as measured by reduction in use of rescue medication, severity of disease or inhibition of the allergen-induced late-phase response[20–22]. In contrast, however, another study using lower doses of *M. vaccae* in asthmatics did not show any beneficial clinical effects[23]. All patients in this study were prescribed inhaled glucocorticosteroids and it is possible that the induction and possible effector function of regulatory T cells might be suppressed by this treatment. Moreover, our studies also demonstrated that allergen exposure is absolutely required to generate allergen-specific regulatory T cells. Therefore, one of the critical elements determining success or failure of a clinical study might be the level and duration of natural exposure to allergens during the treatment period. The new insights into the mechanisms of action of SRP299 treatment as described here should help to design better clinical studies and to understand the dysregulation of the immune response resulting in allergic diseases.

Methods

Animals. Female BALB/c mice (20–25 g, 5–6-wk-old) were obtained from Harlan (Oxon, UK). All experiments complied with the Home Office 1986 Animals Scientific Act and were approved by the NHRC Animal Welfare Committee.

Immunization and airway challenge. Short-term model: mice were immunized intraperitoneally (i.p.) on days 0 and 14 with 10 μg OVA (Grade V, Sigma) or CEA (Hollister-Stier Laboratories, Spokane, Washington) in 0.2 ml of alum (Serva, Heidelberg, Germany). On day 21, OVA-immunized mice were exposed for 20 minutes to an aerosol of OVA (50 mg/ml) or PBS. CEA-immunized mice were challenged with 10 μg CEA in 50 μl PBS or PBS alone administered intranasaly. Long-term model: mice were immunized i.p. on days 0, 14 and 42 and s.c. on day 56 with 10 μg OVA in 0.2 ml of alum and challenged with OVA on day 63 as described above.

Treatment with SRP299. Mice were treated s.c. with 0.1 mg SRP299 (sterile vials containing a suspension of heat-killed *M. vaccae* at 10 mg/ml, SR Pharma) in 200 μl saline or with saline alone three weeks before OVA immunization.

Bronchoalveolar lavage (BAL) fluid. Animals were i.p. anesthetized with 60 mg/kg pentobarbitone sodium. The trachea was cannulated and BAL was collected by injecting 0.4 ml PBS three times into the lung. Total cell count was determined and differential cell counts were performed on cytospin slides stained with Diff-Quik (Baxter Dade AG, Dudingen, Switzerland) counting 200 cells. BAL cytokine levels were assessed using ELISA kits (R&D Systems, Oxon, UK). The detection limits were the following: IL-2 (3 pg/ml), IL-4 (2 pg/ml), IL-5 (7 pg/ml), IL-10 (4 pg/ml), IL-12 (4 pg/ml), IFN-γ (2 pg/ml) and TGF-β (7 pg/ml).

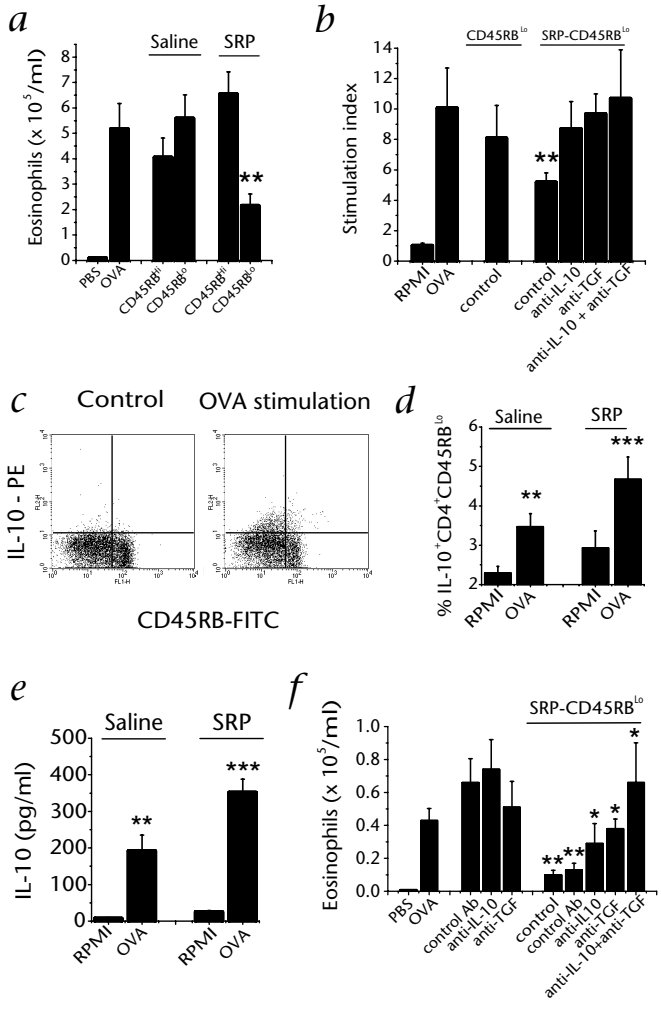


Fig. 3 The inhibitory effect of regulatory T cells resides within the $CD4^+CD45RB^{Lo}$ population and is mediated by IL-10 and TGF-β. ***a***, Adoptive transfer of FACS-sorted $CD4^+CD45RB^{Hi}$ or $CD45RB^{Lo}$ (0.4×10^6 cells/mouse) T cells from saline- (saline) or SRP299-treated (SRP) and OVA-immunized mice into OVA-immunized recipient mice 24 h before PBS (PBS) or OVA (OVA and all other bars) challenge. Data are means ± s.e.m. of 9–12 mice per group. **, $P < 0.05$ versus OVA-challenged mice without cell transfer. ***b***, *In vitro* proliferative response of OVA-stimulated $CD4^+$ T cells from OVA-immunized mice following addition of 1×10^5 purified $CD4^+CD45RB^{Lo}$ T cells from saline-treated ($CD45RB^{Lo}$) or SRP299-treated ($SRP\text{-}CD45RB^{Lo}$) and OVA-immunized mice in the absence (control) or presence of neutralizing anti-IL-10 or anti-TGF-β or a combination. Results are SI (^{3}H-thymidine incorporation induced by OVA/^{3}H-thymidine incorporation in cultures without OVA). Data are means ± s.e.m. of 3 individual cultures. **, $P < 0.05$ versus OVA-stimulated cultures in the absence of cocultured $CD45RB^{Lo}$ cells. ***c***, Representative FACS dot plot of double immunofluorescence stained $CD4^+$ T cells (FITC-conjugated anti-CD45RB antibodies and intracellular staining with phycoerythrin-conjugated anti-IL-10 antibodies) without (control) or following *in vitro* OVA stimulation. ***d***, Quantitative analysis of IL-10 producing, $CD4^+CD45RB^{Lo}$ expressing cells from saline-treated (saline) or SRP299 treated (SRP) and OVA-immunized mice in non-stimulated (RPMI) or OVA (OVA) stimulated cultures. Data are means ± s.e.m. of cultures from 7–10 mice per group. **, $P < 0.05$; ***, $P < 0.05$ versus RPMI and OVA-stimulated cultures from saline-treated mice, respectively. ***e***, IL-10 levels in supernatant from the same cultures as described in *d*. ***f***, OVA-immunized mice were treated with PBS or 0.4×10^6 purified $CD4^+CD45RB^{Lo}$ T cells from SRP-treated and OVA-immunized mice ($SRP\text{-}CD45RB^{Lo}$). In addition, some of the mice were pretreated with either PBS (control), isotype-matched antibodies (control Ab), anti-IL-10, anti-TGF-β or a combination of anti-IL-10 and anti-TGF-β antibodies. BAL fluid was analyzed 48 h after the PBS (PBS) or OVA challenge. Data are means ± s.e.m. of 6–8 mice per group. **, $P < 0.05$ versus OVA-challenged mice without cell transfer and antibody treatment; *, $P < 0.05$ versus $CD45RB^{Lo}$ transfer control.

Cell transfer. 24 h after PBS or OVA challenge (day 22), spleens from saline- or SRP299-treated mice were collected. Single-cell suspensions were prepared by passing cells through a cell strainer. Purified T-lymphocytes, $CD4^+$ or $CD8^+$ fractions were prepared by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, splenocytes were incubated with CD90 (Thy1.2), CD4 (L3T4) or CD8a (Ly-2) microbeads, respectively, and desired cells were separated by positive selection[12]. The resulting populations were >95% pure as determined by FACS analysis. $CD4^+$ T cells were further separated into $CD4^+CD45RB^{Lo}$ and $CD4^+CD45RB^{Hi}$ cells using FITC-conjugated monoclonal antibodies against CD45RB (16A, BD, Oxford, UK) and sorted by flow cytometry (MoFlo, Cytomation). All populations were > 99% pure on reanalysis. 24 h before the antigen challenge (day 20), mice were intravenoulsy (i.v.) injected with 50 μl PBS containing the indicated number of the desired cell population.

***In vitro* cytokine production and proliferation**. Splenocytes from SRP299- or saline-treated, OVA-immunized mice (day 20) were cultured at 2×10^5 cells/culture in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from GIBCO, Paisley, UK) at 37 °C, 5% CO_2, in the presence or absence of 100 μg/ml ovalbumin for 72 h. Supernatants were then collected and cytokine levels determined by ELISA.

Purified $CD4^+$ T cells (2×10^5 cells) were stimulated by OVA (100 μg/ml) in the presence of T cell–depleted, irradiated (4,000 rad) splenocytes. $CD4^+CD45RB^{Lo}$ T cells from SRP299 or saline-treated mice were added to OVA-primed $CD4^+$ T cells in the presence or absence of 60 ng/ml anti-IL-10 (JES052A5, BD) or 10 ng/ml anti-TGF-β (chicken IgY, R&D Systems) antibodies. Proliferation was assessed by [^{3}H]thymidine incorporation (0.5 μCi/well) during the last 16 h of culture and results are expressed as stimulation index (SI, counts of OVA stimulated/counts of non-stimulated cultures).

Intracellular cytokine staining. 2×10^5 splenocytes were cultured with or without OVA for 3 d. For the last 16 h of culture, 3 μM monensin and 5 μg/ml brefeldin A (Sigma) were added. $CD4^+$ T cells were then isolated by positive selection using MACS and stained for CD45RB expression with FITC-anti-CD45RB antibodies. Cells were then fixed, permeabilized (Fix&Perm kit, Caltag) and stained for intracellular IL-10 with phycoerythrin-anti-IL-10 antibody (BD).

Determination of BHR. 24 h after challenge, unrestrained conscious mice were placed in a whole-body plethysmographic chamber (BUXCO, EMKA Technologies). After 2 min stabilization, an aerosol of methacholine (Sigma) at 30 mM was delivered for 1 min. The airway resistance was expressed as Penh (enhanced pause)[24].

***In vivo* treatment with antibody.** Immunized mice (day 20) were injected i.p. with 0.6 mg of rat IgG1 against mouse IL-10, JES052A5, or with its matched isotype control, LO-DNP-2 (provided by M. Goldman) or with 33 μg of chicken IgY anti-TGF-β or with its matched isotype control or with saline, 2 h before the i.v. injection of 4×10^5 $CD4^+CD45RB^{Lo}$ T cells. BAL was performed 48 h post-challenge.

Statistical analysis. Data were analyzed using microcomputer programs for one-way ANOVA followed by Student's *t*-test for unpaired values. $P < 0.05$ was considered significant. Results are expressed as means ± s.e.m.

Acknowledgments

We thank J. Lee, A. Nicholls and G. Dubois for technical support.

Competing interests statement

The authors declare competing financial interests: see the website (http://medicine.nature.com) for details.

RECEIVED 4 FEBRUARY; ACCEPTED 1 APRIL 2002

1. Bousquet, J., Jeffery, P.K., Busse, W.W., Johnson, M. & Vignola, A.M. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* **161**, 1720–1745 (2000).
2. Martinez, F.D. & Holt, P.G. Role of microbial burden in aetiology of allergy and asthma. *Lancet* **354** (suppl. 2), 12–15 (1999).
3. Shirakawa, T., Enomoto, T., Shimazu, S. & Hopkin, J.M. The inverse association between tuberculin responses and atopic disorder. *Science*, **275**, 77–79 (1997).
4. Farooqi, I.S. & Hopkin, J.M. Early childhood infection and atopic disorder. *Thorax*, **53**, 927–932 (1998).
5. Erb, K.J., Holloway, J.W., Sobeck, A., Moll, H. & Le Gros, G. Infection of mice with Mycobacterium bovis-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J. Exp. Med.* **187**, 561–569 (1998).
6. Herz, U. *et al.* BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J. Allergy Clin. Immunol.* **102**: 867–874 (1998).
7. Gajewski, T.F. & Fitch, F.W. Anti-proliferative effect of IFN-γ in immune regulation: IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T-lymphocyte clones. *J. Immunol.* **140**, 4245–4252 (1988).
8. Stene, L.C. & Nafstad, P. Relation between occurrence of type 1 diabetes and asthma. *Lancet* **357**, 607 (2001).
9. Wang, C.C. & Rook, G.A. W. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed *Mycobacterium vaccae*. *Immunology* **93**, 307–313 (1998).
10. Roncarolo, M.G. & Levings, M.K. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol.* **12**, 676–683 (2000).
11. Groux, H. *et al.* A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737–742 (1997).
12. Zuany-Amorim, C. *et al.* Modulation by IL-10 of antigen-induced IL-5 generation and $CD4^+$ T lymphocyte and eosinophil infiltration into the mouse peritoneal cavity. *J. Immunol.* **157**, 377–384 (1996).
13. Maloy, K.J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nature Immunol.* **2**, 816–822 (2001)
14. Thornton, A.M. & Shevack, E.M. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T-cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296 (1998).
15. Levings, M.K., Sangregorio, R. & Roncarolo, M.G. Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T-cell proliferation and can be expanded *in vitro* without loss of function. *J. Exp. Med.* **193**, 1295–1301 (2001).
16. Roncarolo, M.G., Levings, M.K. & Traversari, K. Differentiation of T regulatory cells by immature dendritic cells. *J. Exp. Med.* **193**, F5–F10 (2001).
17. Cottrez, F., Hurst, S.D., Coffman, R.L. & Groux, H. T regulatory cells 1 inhibit a Th2–specific response *in vivo*. *J. Immunol.* **165**, 4848–4853 (2000).
18. Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1004 (1999).
19. Davies, J.D. *et al.* $CD4^+CD45RB$ low-density cells from untreated mice prevent acute allograft rejection. *J. Immunol.* **163**, 5353–5357 (1999).
20. Hopkins, J.M. Mycobacterial immunization: agents to limit asthma. in *New Drugs for Asthma, Allergy and COPD* (eds. Hansel, T.T. & Barnes, P.J.) 226–228 (Karger, Basel, Switzerland, 2001).
21. Arkwright, P.D. & David, T.J. Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J. Allergy Clin. Immunol.* **107**, 531–534 (2001).
22. Camporota, L. *et al.* Effects of intradermal injection of SRL172 (killed *Mycobacterium vaccae* suspension) on allergen-induced airway response and IL-5 generation by PBMC in asthma. *Am. J. Resp. Crit. Care Med.* **161**, A477 (2000).
23. Shirtcliffe, P.M. *et al.* The effect of delipidated deglycolipidated (DDMV) and heat-killed *Mycobacterium vaccae* in asthma. *Am. J. Respir. Crit. Care Med.* **163**, 1410–1414 (2001).
24. Zuany-Amorim, C. *et al.* Requirement for γδ T cells in allergic airway inflammation. *Science*. **280**, 1265–1267 (1998).