

The effect of endotoxin on the production of IgE, IgG1 and IgG2a antibodies against the cat allergen Fel d 1 in mice

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Abstract

Background: Endotoxin/LPS is ubiquitous in our environment. The question whether lipopolysaccharide (LPS) is beneficial or disease-promoting in relation to asthma and allergy has been raised in several recent studies. Some have reported a positive correlation between the level of LPS in house dust and the symptoms of asthmatic children. Others have found that exposure to LPS appears to protect against the development of atopic disease in children. **Objectives:** We performed a study in mice to examine the antibody response after subcutaneous immunization with LPS and the cat allergen Fel d 1. We asked whether LPS would increase the response and direct the antibody production towards an allergic (IgE), or non-allergic (IgG2a) antibody profile. In rodents both IgE and IgG1 are antibodies produced under Th2-dependence and IgG2a antibodies under Th1-dependence. Also, when LPS and Fel d 1 are introduced to the immune system, we asked whether the timing of the two agents relative to each other is crucial. **Methods:** The mice were injected subcutaneously with LPS and/or Fel d 1 four times in various orders. IgE, IgG1 and IgG2a antibodies specific to Fel d 1 were measured in serum using ELISA. **Results:** A strong antibody response, both for IgE, IgG1 and IgG2a, was observed only when Fel d 1 and LPS were injected simultaneously, and in particular after repeated injections. **Conclusion:** A strong specific antibody response was observed, both for IgE, IgG1 and IgG2a, only when LPS was introduced to the immune system together with the cat allergen Fel d 1. No such adjuvant effect was observed when LPS was introduced alone prior to or subsequent to the allergen. The resulting antibody response was not polarized in terms of Th1- or Th2-dependence.

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1. Introduction

Many studies from all over the world indicate an increase in prevalence of asthma and allergy during the last decades (Mushinski, 1997; Jarvis and Burney, 1998; Sly, 1999). The underlying causes remain unclear. Asthma and allergy are

Abbreviations: LPS, lipopolysaccharide.

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influenced by many genetic and environmental factors. Many different factors regarding lifestyle and environment have been studied, such as socioeconomic status, family size, nutrition, infections, allergen exposure, and last but not least, different air pollution components (reviewed by von Mutius et al., 2000). Much focus has lately been set on the indoor environment, as it has been estimated that the Western populations today spend 95% of their time indoors (Platts-Mills et al., 1995). Microbial exposure in the indoor environment is of great interest in relation to increased prevalence of allergy.

Gram-negative bacteria and their endotoxins are ubiquitous in nature, and endotoxin/lipopolysaccharide (LPS) has been measured in house dust (and outdoor air particulate matter) by several groups all over the world (Michel et al., 1996; Wan and Li, 1999; Park et al., 2000; Douwes et al., 2000; von Mutius et al., 2000). In addition to house dust, endotoxins can also be found in tap water, and in milk (Lapa e Silva et al., 2000). Thus, everyone is exposed to some level of endotoxin. Endotoxin has been found to profoundly influence the immune system and body homeostasis.

Differentiation of T-helper ($CD4^+$) cells into two subsets, Th1 and Th2, each with a characteristic profile of cytokine production, is central to the understanding of the pathogenetic mechanisms of allergy. It seems clear that LPS in general shifts the cytokine balance towards a Th1 response. It is found to induce $IFN-\gamma$ and IL-12 in human peripheral blood cells in vitro (Le et al., 1986; D'Andrea et al., 1992), and other laboratory studies support this impression of LPS being a Th1-inducer (reviewed in Lapa e Silva et al., 2000). According to the concept that Th1- and Th2-responses reciprocally counteract each other, this should mean that LPS may down-modulate Th2-type immune development, and consequently counteract the development of allergy and allergy related asthma. This possibility has been discussed as one of several possible explanations why children raised in farming communities apparently have less allergic rhinitis and allergen sensitization than other children (Braun-Fahrlander et al., 1999; von Ehrenstein et al., 2000; Riedler et al., 2000; Kilpeläinen et al., 2000). von Mutius et al. (2000)

measured and compared the concentrations of endotoxin in house dust from farming and non-farming families. They found that the endotoxin concentrations were significantly higher in the houses of the farming families, as compared with nonfarming families.

In contrast to these findings of LPS being (partially) protective in relation to atopy, several studies indicate that LPS may worsen asthma. Some investigators have reported a positive correlation between the level of LPS in the house dust and symptoms in asthmatic children (Michel et al., 1991; Bjornson et al., 1995; Michel et al., 1996; Rizzo et al., 1997). Furthermore, several laboratory studies in humans indicate that LPS challenge may influence immunological factors leading to the worsening of asthma (Peden et al., 1999; Eldridge and Peden, 2000).

These findings regarding the influence of endotoxin/LPS on allergy development and asthma are not necessarily conflicting. The explanation could lie in the timing of the exposure of allergen and LPS, that is, the clue may be whether an allergen sensitivity exists prior to the LPS exposure or not.

The cat allergen Fel d 1 is an important indoor allergen (often found also in homes without a cat), and experimental exposure to this allergen together with LPS should, therefore, be of relevance for a real-life environmental factor interaction. The combination of LPS and Fel d 1 is perhaps of special interest, as the cat must be assumed to bring extra endotoxin into the home (Gereda et al., 2001). We performed a study to examine whether LPS would increase the response and direct the antibody response into an allergic (IgE) or non-allergic (IgG2a) antibody profile. Furthermore, we asked whether it is crucial, concerning the resulting type of antibody response (Th1- or Th2-dependent), when LPS and allergen are introduced to the immune system relative to each other. In mice, the most important signature cytokine of Th2 cells, IL-4, induces class switch in B-cells to the antibody classes IgE and IgG1. $IFN-\gamma$, from Th1 cells, stimulates the production of IgG2a antibodies (Coffman et al., 1993). We, therefore, measured the serum levels of IgE, IgG1 and IgG2a antibodies specific to Fel d 1 in the serum of mice

exposed to LPS and Fel d 1 in various sequential orders.

2. Methods

2.1. Mice

Female Balb/cA mice (6–8-week old) were obtained from B&M Ltd., Ry, Denmark., and were rested for 1 week after arrival. The mice were kept in Scantainer filter cabinets in Makrolon Type 3 cages on BeeKay Bedding (B&K Universal, Nittedal, Norway), eight mice per cage. They were given food (expanded rat and mouse no. 1 Maintenance diet, RM1 (E) manufactured by SDS (Essex, UK)), and tap water ad libitum. The experiments were approved by the local officer of the Animal Board under the Ministry of Agriculture in Norway and performed in conformation with the laws and rules regulating animal experiments in Norway.

2.2. Purification of Fel d 1 allergen

The major cat allergen, Fel d 1, was purified from cat dander by sequential monoclonal antibody (mAb) affinity chromatography and HPLC

size exclusion, as described in more detail elsewhere (Duffort et al., 1991).

2.3. Preparations for immunization

Hank's balanced salt solution (HBSS; Gibco BRL, Paisley, Scotland) was used as medium. LPS was from *Escherichia coli* (026:B6, Sigma, St Louis, MO, USA) and was suspended in medium (0.5 mg/ml). Freeze dried Fel d 1 was suspended in medium (0.5 mg/ml). These batch solutions were diluted 1:2 either with medium (to make solutions of LPS or Fel d 1 alone) or with each other (to make LPS+Fel d 1), to prepare the different solutions for immunization. The dose of LPS and Fel d 1 was 5 µg each in 20 µl immunization solution.

2.4. Experimental design

Groups of eight mice were injected with LPS and/or Fel d 1 or medium at different time points (Table 1). The mice were injected with 20 µl of the different immunization suspensions in the right hind footpad (heel–toe direction), using a 100 µl Hamilton syringe (Hamilton Bonaduz AG, Switzerland) fitted with a 30 G needle (Becton, Dickinson and Co, Dublin, Ireland). The mice were unanesthetized during the injections. Three

Table 1
Experimental groups

Group	Day					
	–10	–3	0	20	21	26
A	LPS	Fel d 1	Fel d 1	Blood sample drawn	Fel d 1	Exsanguination
B	LPS	LPS/Fel d 1	Fel d 1			
C	LPS	LPS	LPS/Fel d 1			
D	LPS/Fel d 1	LPS	Fel d 1			
E	LPS/Fel d 1	LPS	LPS			
F	LPS/Fel d 1	LPS/Fel d 1	LPS/Fel d 1			
G	Fel d 1	Fel d 1	LPS			
H	Fel d 1	Fel d 1	Fel d 1			
I	Med.	Med.	LPS/Fel d 1			
J	Med.	Med.	LPS			
K	Med.	Med.	Fel d 1			
L	Med.	Med.	Med.		Med.	

priming phase injections were given on days -10, -3 and 0, and blood samples were drawn from the distal part of the lateral femoral vein on day 20 to measure serum levels of antibodies. The blood sampling procedure has been described in more detail elsewhere (Aaberge et al., 1992). On day 21 all groups of mice were boosted with Fel d 1. On day 26 the mice were exsanguinated under CO₂ anesthesia, and serum from each mouse was prepared and frozen for later measurement of Fel d 1 specific IgE, IgG1 and IgG2a antibodies using ELISA.

2.5. Biotinylation of Fel d 1 for use in ELISA

Biotin-X-NHS (Calbiochem, La Jolla, CA, USA) was suspended in DMSO (Sigma) (1 mg/ml). Freeze dried Fel d 1 allergen was suspended in 0.1 M NaHCO₃ with 0.5 M NaCl, pH 8.3 (500 µg/ml). Four hundred µl allergen solution + 40 µl 0.1 M NaHCO₃ with 0.5 M NaCl, pH 8.3 + 60 µl biotin-X-NSH was mixed well in an Eppendorf tube and incubated at room temperature for 2 h. The solution was then dialyzed in PBS/NaN₃, with change of PBS/NaN₃ two times at 2-h intervals, before dialysis overnight at 4 °C with PBS/NaN₃. The quality of the biotinylation of the Fel d 1 was tested in a dot blot.

2.6. Assay for serum Fel d 1-specific IgE, IgG1 and IgG2a antibodies

Polystyrene microtiter plates (Immulon II M 129B, Dynatech Laboratories Ltd., Chantilly, VA, USA) were coated with 100 µl of 2 µg/ml rat anti-mouse IgE (clone LOME-3, Serotec, Oxford, UK), rat anti-mouse IgG1 (clone LO-MG1-13, Serotec Ltd.), or rat anti-mouse IgG2a antibody (clone LO-MG2a-7, Serotec Ltd.) in 0.05 M carbonate/bicarbonate buffer, pH 9.6. The coated plates were incubated for 1 h at room temperature and thereafter at 4 °C overnight. After five washes with Tris/Tween the plates were incubated with a Fel d 1-specific standard serum and the sera to be tested diluted in BSA/Tris/Tween, 100 µl in each well. Duplicates of a 1:20 dilution for IgE and IgG2a, and a 1:100 dilution for IgG1 were used. The plates were then incubated for 2 h at room

temperature followed by 4 °C overnight. After five new washes with Tris/Tween, the plates were incubated for 2 h at room temperature with an optimal concentration of biotin labeled Fel d 1 (0.5 µg/ml) in Tris/Tween, 100 µl per well. After another washing procedure, the plates were incubated for 1 h at room temperature with alkaline phosphatase conjugate (Extr-Avidin, Sigma). Again the plates were washed, and incubated at room temperature with 100 µl *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate, Sigma) in 10% diethanolamide buffer pH 9.8. The plates were read at 405 nm in a MRX Microplate Reader (Dynatech Laboratories, UK). The amount of specific antibodies was expressed as arbitrary units.

2.7. Statistical analysis

One way ANOVA analysis for multiple comparisons was used. Pairwise comparisons were made with Student's *t*-test when there was a Gaussian distribution of the results and otherwise by the non-parametric Mann–Whitney *U*-test. *P* values < 0.05 were considered statistically significant.

3. Results

The mice were injected with Fel d 1, LPS, or the combination thereof, with medium as a control exposure (Table 1). The simplest priming situation is represented by groups I, J, K and L. These groups were given, without any priming other than medium, Fel d 1/LPS, LPS, Fel d 1 or medium, respectively, on day 0 (Table 1). No statistically significant specific IgE, IgG1, or IgG2a response was detected on day 20 (Fig. 1a–c, respectively).

However, the effect of this priming on the secondary response, as measured after Fel d 1 boosting on day 21, was strikingly different for the different groups. Simultaneous exposure to Fel d 1 and LPS (group I) primed for a specific IgE secondary response that was among the strongest observed in the experiment (Fig. 2a), and also an IgG2a response that together with the response in group F (repeated instead of single Fel d 1/LPS

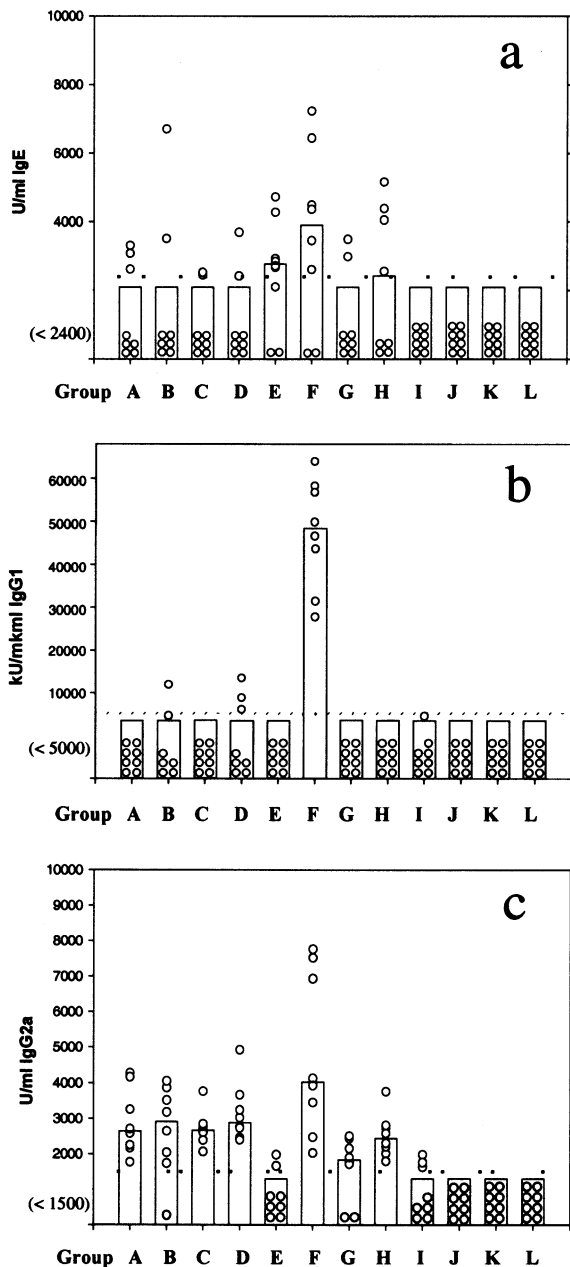


Fig. 1.

priming) was by far the strongest observed (Fig. 2c). An IgG1 response was also detected (Fig. 2b). Fel d 1 alone (group K), in contrast, did not prime for a detectable secondary response in any of the three isotypes measured, nor did LPS alone (group

J). Thus, simultaneous exposure to Fel d 1 and LPS by far represented the most effective priming (group I). The secondary response was strong both in the Th2-dependent IgE and IgG1 isotypes (Fig. 2a and b), and the Th1-dependent IgG2a isotype in this group (Fig. 2c). IgE and IgG2a showed about the same increase in antibody levels compared with the control groups, that is five and eight times higher, respectively (Fig. 2a and c). IgG1 showed the strongest relative increase in this combination group, that is, 14 times over the controls (Fig. 2b).

Priming exposure to Fel d 1, given three times (group H), did not give a secondary response different from a single priming dose of Fel d 1 (group K) for any isotype (Fig. 2a–c). Substitution of the first dose or the last dose of Fel d 1 with LPS (group A or G, respectively), as compared with group H (Fel d 1 three times), had no effect on the secondary response. On day 20, however, a small, but statistically significant, reduction in the IgG2a level was observed in group G compared with group H.

Most interestingly, three times priming with the combination Fel d 1/LPS (group F) induced responses in all isotypes that were among the strongest observed on day 20, and especially in the secondary response, as measured on day 26. In the secondary response, IgE and IgG2a showed about the same relative increase in antibody levels compared with group H, that is five and eight times higher, respectively (Fig. 2a and c). IgG1 showed a tremendous increase in the three times primed combination group, reaching levels 100 times higher than in group H (Fig. 2b).

It is interesting to notice how the effect from three-times-combination priming (group F) was

Fig. 1. Levels of specific anti-Fel d 1 antibodies in serum on day 20 after subcutaneous injection in the footpad with 5 µg LPS, 5 µg Fel d 1, or medium three times (days -10, -3 and 0) in various combinations (see Table 1). Values for individual mice and median values for groups of eight mice (columns) are given. The antibody levels are expressed as arbitrary units. The dashed line indicates the lower detection limit for anti-Fel d 1-specific antibodies in the ELISA used. The following anti-Fel d 1 isotypes are shown: (a) IgE, (b) IgG1 and (c) IgG2a.

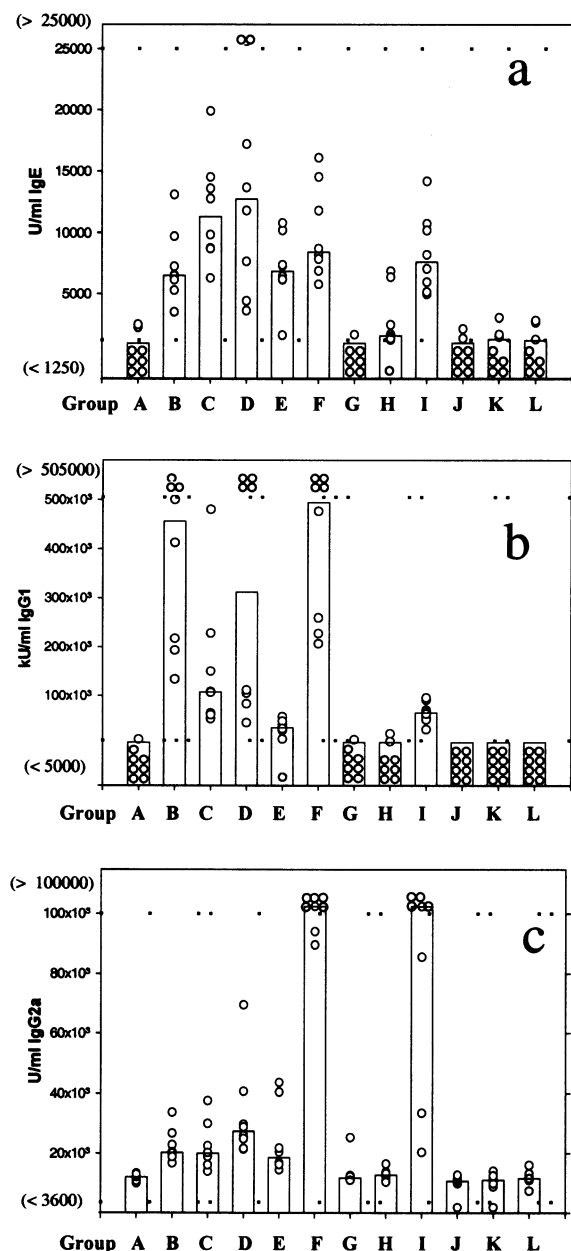


Fig. 2.

modified by different substitutions. Substitution of the two last priming doses (group E) with LPS significantly reduced the antibody responses, both on day 20 and in the secondary response. In particular, the IgG1 and IgG2a isotypes were strongly reduced. Substitution of the two first

priming doses by LPS alone (group C), also reduced the antibody responses, compared with group F, except for the IgE level on day 26. Substitution of the middle priming dose by LPS alone and the last one by Fel d 1 (group D), showed similar results as group C. Substitution of the first dose by LPS alone and the last dose by Fel d 1 alone (group B), also reduced the antibody responses compared with group F, except for the IgG1 level on day 26.

When it comes to the effect of LPS alone, it was found that it made no difference whether LPS was given prior to (group A) or subsequent to (group G) a two-dose priming of Fel d 1. However, if a single combination dose of Fel d 1 and LPS was preceded by LPS (group C), this altered the response compared with pretreatment with medium only (group I). On day 20, the IgG2a level was increased in group C, as compared with group I. In the secondary response, however, the IgG2a level was clearly reduced by the LPS pretreatment (group C vs. group I). In contrast, the IgE and IgG1 responses tended to be moderately increased in group C, as compared with group I (not statistically significant).

Thus, in the experimental model used, by far the strongest response, in all three antibody classes, was elicited by the combination of Fel d 1 and LPS, and in particular by repeated exposures to this mixture (group F). Two times priming with Fel d 1 alone, preceded (group A) or followed (group G) by LPS, did not show any difference in the antibody response from three times priming with Fel d 1 (group H) in the secondary response. However, on day 20, a significantly reduced IgG2a level was seen when the last of three Fel d 1 priming doses was substituted by LPS.

Fig. 2. Levels of specific anti-Fel d 1 in serum day 26 after three priming injections in the footpad with 5 μ g LPS, 5 μ g Fel d 1 or medium (days -10, -3 and 0), and a booster dose of 5 μ g Fel d 1 on day 21 (see Table 1). Values for individual mice and median values for groups of eight mice (columns) are given. The antibody levels are expressed as arbitrary units. The dashed lines indicate the upper and the lower detection limits for IgE a-Fel d 1 antibodies in the ELISA used. The following anti-Fel d 1 isotypes are shown: (a) IgE, (b) IgG1 and (c) IgG2a.

A repeat experiment gave essentially similar results.

4. Discussion

All groups (except the medium control group) were boosted with Fel d 1 on day 21. The secondary response data will, therefore, allow the comparison of the effect on the secondary response of different priming regimen. The priming regimen were set up to model different sequences of exposure to Fel d 1 and LPS, that is, simultaneous exposure, or one substance before the other.

Priming once with the combination of Fel d 1 and LPS (group I) resulted in strongly elevated levels of all three antibody classes, compared with priming once with Fel d 1 alone (group K). Thus, LPS had an adjuvant effect on the antibody production to the allergen Fel d 1, when given simultaneously with Fel d 1. Most interestingly, the adjuvant effect not only included the Th2-dependent antibody isotypes IgE and IgG1, but also the Th1-dependent IgG2a isotype. This contrasts what we have previously found with some other environmental factors using footpad immunization, like diesel exhaust particles (DEP) (Løvik et al., 1997) and β -glucan (Ormstad et al., 2000). This means that LPS, in contrast to some other environmental components, helps induce an isotypically broad antibody response, including both Th2- and a Th1-dependent antibody responses at the same time.

The strong antibody response observed was elicited only by the combination of Fel d 1 and LPS. In particular, repeated exposures to the combination of Fel d 1 and LPS (group F) gave noticeably strong antibody responses, both on day 20, as well as in the secondary response measured on day 26. This adjuvant effect of LPS was not seen when LPS was given separately from Fel d 1. It made no difference whether LPS was given prior to or subsequent to two priming doses of Fel d 1 (groups A and G, respectively). However, if a single combination dose of Fel d 1 and LPS was preceded by two priming doses of LPS (group C), a clearly reduced IgG2a and slightly increased IgE and IgG1 responses were seen in the secondary

response, as compared with mice in which the combination dose was preceded by two injections with medium only (group I) (Fig. 2a–c). This indicates that the response was skewed in the direction of a weaker Th1-dependent response, and a slightly elevated Th2-dependent antibody response when the immune system was introduced (twice) to LPS before the combination dose of LPS and Fel d 1.

In the mouse model used, the simultaneous introduction of LPS and allergen to the immune system, gave a strong allergen specific antibody response in both Th1 and Th2-dependent isotypes. LPS given alone did not alter the antibody response to the allergen given alone, neither if LPS was given prior to or subsequent to the allergen. Similar findings, in various mouse models (Danneman and Michael, 1976; Seyka, 1983; Jiang et al., 1998; Slater et al., 1998; Wan et al., 2000) or in Guinea-pigs (Perini and Mota, 1973), have also been reported earlier. This suggests that no polarization towards Th1- or Th2-dependent response occurs after LPS and allergen exposure in mice. This is in contrast to what has been claimed to be the case in humans, namely that LPS is a Th1-inducer (Le et al., 1986; D'Andrea et al., 1992; reviewed in Lapa e Silva et al., 2000). Gereda et al. (2000) examined 61 infants 9–24 months old and found that the homes of allergen-sensitized (skin-prick positive) infants contained significantly lower concentrations of house dust endotoxin than those of non-sensitized infants. They also found that housedust endotoxin concentrations correlated with increased proportions of IFN- γ producing T-cells, while no correlation was found with cells producing IL-4, IL-5 or IL-13. These correlations, therefore, suggested that indoor endotoxin exposure early in life may protect against allergen sensitization by enhancing type 1 immunity. However, the possibility remains that LPS is only a marker for some other causative factor, e.g. the microbes themselves.

Even if LPS enhances type 1 immunity, the clinical status of some allergic diseases may be worsened because LPS will increase inflammation (Peden et al., 1999; Michel et al., 2001). This means that LPS can augment the morbidity in individuals with already established airways dis-

ease, although it seems to protect against the development of allergic immune responses prior to disease onset, as discussed by Liu and Leung (2000). Tulic et al. (2000) found, in rats, that a single dose of inhaled LPS, either before or within the first 4 days of sensitization to ovalbumin, abolished the symptoms of asthma, and also decreased IgE levels, but increased IgG levels, as compared with ovalbumin given alone. In contrast, if LPS was given from day 6 and later, augmented airway hyperresponsiveness and inflammation were observed, and also increased IgE levels, but decreased the IgG levels. Thus, when LPS is given prior to or during the sensitization phase, it seems that a Th1-, but not a Th2-response will result. In contrast, when LPS is given later, after a Th2-response has been established, LPS will not affect the Th1/Th2 polarization. The already established Th2 response will then prevent the development of Th1 cells facilitated by LPS. Finally, our results indicate that after a simultaneous exposure of LPS and allergen, with LPS and Fel d 1 being introduced at exactly the same time, both a strong Th1 and a strong Th2 response will develop simultaneously. It seems like, in that case, the two oppositely regulated responses do not prevent each other and develop in parallel. A simultaneous exposure to LPS and allergen represents most likely the real-life exposure in humans. However, the apparent Th1 polarization in humans is not explained by our findings.

There are several possible explanations for the different findings in humans and mice. It can not be excluded that the immune systems of humans and mice respond differently to LPS and allergen. Besides, the Th1/Th2 polarization model is most clearly defined in mice. In humans the difference and relationship between Th1 and Th2 cells is less clearly defined (Frew, 1996), although there are many indications for the presence of Th2-like cells in allergic asthmatic airways. Besides, marked differences in the airway responsiveness to inhaled LPS in humans have been found (Kline et al., 1999), indicating that individuals have an unique physiologic response to inhaled endotoxin.

Further, there are several factors in the mouse models used that differ from the real-life situation in humans. We used subcutaneous injection in the

mice, and therefore, did not stimulate the mucosal immune system of the airways. Erb et al. (1998) found that BCG more effectively gave a Th1 response when given into the nasopharynx versus subcutaneously. However, Slater et al. (1998) used nasal instillation in mice, and still did not find any polarization towards Th1 or Th2 when LPS and allergen were given together. Another difference between the mouse model used and the human situation, is that in the human situation there will be a more continuous exposure to LPS (and allergen). In the mouse model used, we gave bolus exposures to LPS and allergen. This difference is not unlikely to affect the quality of the immune response. In fact, it has been found, also in humans, that bolus (intravenous) exposure to LPS shifts the cytokine response towards a Th2 type (Zimmer et al., 1996).

Another factor that may affect the immune response seen is the dose of LPS. No LPS dose-effect was tested in this study.

In summary, we found that a strong specific antibody response was induced, both for IgE, IgG1 and IgG2a, only when LPS was introduced to the immune system simultaneously with the cat allergen Fel d 1. Thus, the resulting antibody response was strong both for Th1- and Th2-dependent isotypes, that is, no polarization was found after LPS exposure in our mouse model. Simultaneous exposure to varying amounts of LPS and allergen is presumably the most common way of exposure in man. Our findings indicate that LPS may aggravate allergic airway disease. Perhaps most important, individuals not allergic before, may start producing IgE and IgG antibodies to allergens and thereby possibly develop allergy. Furthermore, in addition to causing non-specific inflammation, the enhanced IgE and IgG antibody production caused by LPS may increase the inflammatory response in already allergic airways.

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