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Low-dose endotoxin in allergic asthmatics: effect on bronchial and inflammatory response to cat allergen

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Summary

Background Endotoxin was proposed to increase the severity of asthma. Endotoxin levels greatly differ according to settings. In domestic environments, airborne concentrations may be dramatically low compared with levels reported in occupational settings.

Objective Our first objective was therefore to assess the effect of inhalation of low-level lipopolysaccharide (LPS) on the immediate and late-phase asthmatic bronchial response. Our second objective was to evaluate the effect of exposure to LPS on the local and systemic inflammatory response.

Methods Nineteen asthmatics sensitized to cat underwent on two separate occasions a bronchial challenge test to cat allergen (cat BCT) preceded randomly by a pre-exposure to either saline or LPS (2 µg). Methacholine challenge test was performed 24 h before exposure to LPS or saline. The Borg scale for dyspnoea and lung function were recorded before and after exposure to LPS or saline, and before and after cat BCT. Induced sputum and blood samples were collected before and after cat BCT, and analysed for cell counts and eosinophil cationic protein (ECP) levels.

Results Inhalation of 2 µg LPS did not induce any changes in forced expiratory volume in 1 s (FEV₁), peak expiratory flow (PEF), FEF 25–75 and Borg scale of dyspnoea. It neither modified FEV₁ PD₂₀ (45.03 ng as compared with 87.03; *P* = 0.42). As well, there was no significant difference in late-phase reaction. Pre-exposure to LPS did not influence eosinophil counts or ECP levels in blood and sputum.

Conclusion Our study demonstrated that pre-exposure to LPS at low levels, which may be encountered in domestic environment, had no significant effect on the immediate and late-phase bronchial response to cat allergen. It neither modified local and systemic eosinophilic inflammation.

Keywords asthma, bronchial challenge test, cat allergen, endotoxin, inflammation, lipopolysaccharide

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Introduction

Endotoxin, a lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria, is a common dust and air contaminant in various farming (grain and swine confinement facilities and poultry farms) and industrial (cotton mill, potato processing and animal feed industry) environments [1–3]. In such settings, exposure to endotoxin has been associated with the development of respiratory symptoms, including airway obstruction and hyper-responsiveness, suggesting that endotoxin may exacerbate asthma [4].

In agreement with this hypothesis, experimental exposure to high doses (20–200 µg) of endotoxin has been reported to induce bronchospasm in both asthmatic and non-asthmatic subjects [5, 6]. Furthermore, subjects with allergic asthma were shown to be more sensitive to the bronchoconstrictive effect of endotoxin [5]. Endotoxin exhibits potent pro-inflammatory activity when inhaled and this activity may contribute to increase asthma severity [7].

Endotoxin has been found in domestic environments and epidemiological studies have suggested that domestic exposure to endotoxin could exacerbate asthma. Indeed,

in adult and children asthmatics sensitized to house dust mite, asthma severity was better correlated with endotoxin concentration in house dust rather than mite allergen concentration [8, 9]. However, airborne endotoxin levels found in home settings may be dramatically low. Indeed, whereas airborne endotoxin concentrations reaching up to 1340 ng/m³ were measured in occupational settings [3], levels found in homes varied from 0.065 to 3 ng/m³ [10, 11]. Likewise, we measured airborne endotoxin concentrations of 0.18 ng/m³ in dwellings of Strasbourg's metropolitan area, demonstrating that in urban homes endotoxin levels may be 100–1000 times lower than those measured in air of occupational settings [12]. Interestingly, we also found that these concentrations were not correlated with levels in dust [12].

Therefore, to investigate the role of domestic exposure to endotoxin in allergic asthma, we assessed the effect of pre-exposure to low-dose endotoxin on immediate and late-phase asthmatic bronchial response, as well as on local and systemic eosinophilic inflammation.

Methods

Patients

Patients were enrolled in the study after giving written informed consent. Inclusion criteria were a history of cat

allergic intermittent asthma with a positive skin prick test (mean diameter of weal equal to or greater than 75% of the mean diameter of the positive control, 10 mg/mL histamine) to cat allergen extracts (Stallergènes Laboratory, Fresnes, France) and specific IgE to cat allergens higher than 0.75 kU/L (Pharmacia Laboratory, Uppsala, Sweden). None of the patients had cats at home or any contact with cats, and none had symptoms or a respiratory tract infection within the 2 weeks that preceded the study. Patients were non smokers (12 out of 19) or low (less than five cigarettes per day) smokers. They only used short-acting β_2 agonists on demand.

Study design

It was a randomized, crossover, double-blind protocol (Fig. 1). Each patient underwent on two separate occasions a bronchial challenge test to cat allergen (cat BCT), preceded by a pre-exposure to either sterile saline or LPS. The two sessions were separated by a washout period of no less than 3 weeks. The protocol was approved by the ethical committee of the hospital.

Bronchial challenge tests to methacholine

Non-specific bronchial responsiveness (NSBR) to methacholine was assessed 24 h before exposure to either LPS or saline. BCT to methacholine were performed using a

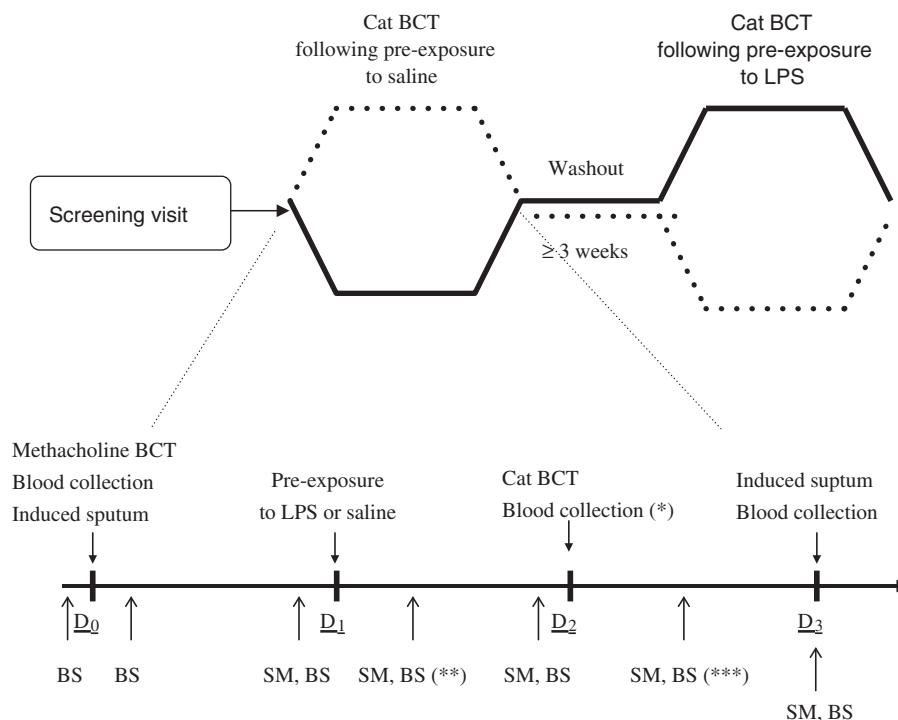


Fig. 1. Study design. Each patient underwent on two separate occasions a bronchial challenge to cat allergen preceded randomly by a pre-exposure to either saline or lipopolysaccharide (LPS; 2 µg). The two protocol sessions were separated by a washout period of no less than 3 weeks. SM, spirometry. BS, Borg scale. *At 3 and 6 h after bronchial challenge test to cat allergen (cat BCT). **At 15, 30, 45, 60, 90, 120, 240 and 360 min after pre-exposure to saline or LPS. ***At the end of cat BCT, and 3 and 6 h later.

dosimeter (Mediprom FDC 88, Paris, France) that administered doubling doses of a 1% methacholine solution from 25 to 3200 µg (cumulative), as previously described [13]. Inhalations of methacholine were stopped when forced expiratory volume in 1 s (FEV₁) dropped by at least 20% of the baseline value within 15 min. The results were expressed as the dose of methacholine inducing a 20% decrease in FEV₁ (methacholine PD₂₀).

Exposure to lipopolysaccharide

Exposure to LPS was carried out 24 h before cat BCT. LPS (*Escherichia coli*, serotype 026:B6, Sigma Chemical, St. Louis, MO, USA) was dissolved in sterile saline and nebulized with an automatic inhalation-synchronized dosimeter (Spira Elektro 2, Hameenlinna, Finland) connected to a nebulizer (Pari Tia; Pari Aerosol Research Institute, Munich, Germany), which delivered particles of a mass median aerodynamic diameter (MMAD) of 10 µm [13]. Each patient performed seven successive inhalations of an LPS solution (50 µg/mL) through the mouthpiece from the residual volume (RV) to total lung capacity (TLC), a nose clip in place. The total dose of inhaled LPS was 2 µg. This dose corresponded to 1/10 of the dose previously used in the bronchial provocation test in asthmatics [5]. Clinical examination, blood pressure, body temperature, dyspnoea Borg scale and spirometry were performed before and 15, 30, 45, 60, 90, 120, 240 and 360 min after the nebulization.

Bronchial challenge tests to cat allergens

Cat BCTs were performed as previously described [13], using the same device as the one used for exposure to LPS. Particles delivered by the nebulizer had an MMAD of 10 µm. Cat BCTs were carried out by inhalation (2, 4 and 8 breaths) of increasing concentrations (10⁻⁴–10 IR) of cat allergen extracts (Stallergènes Laboratories, Fresnes, France, 100 Indice de Réactivité (IR), 13 µg/mL Fel d 1). The starting concentration of cat allergens was determined for each patient, as the end point/100 of prick test scale. The challenge was stopped when the FEV₁ dropped by 20% or more within 15 min. The provocative dose of cat allergen was calculated as the dose of allergen extracts triggering a 20% fall in FEV₁ (Fel d 1 PD₂₀). At the end of the test, subjects inhaled β₂-agonist as necessary and remained under observation for at least 6 h. The Borg scale for dyspnoea was measured before cat BCT, at the end of the cat BCT, and 3, 6 and 24 h later. There were no measurable endotoxin levels in cat allergen extracts, as assessed by the Limulus amoebocyte test.

Blood and sputum collection

Blood and sputum were collected for determination of cell counts and eosinophil cationic protein (ECP) levels. Blood

was collected before exposure to LPS or saline (baseline) and 3, 6 and 24 h after cat BCT. It was then immediately processed for cell counting or incubated for 1 h at room temperature for serum preparation. Sputum were collected before pre-exposure to LPS or saline and 24 h after cat BCT, according to the method described by Pin et al. [14]. After administration of 200 µg salbutamol, subjects inhaled sterile saline at increasing concentrations (3%, 4% and 5%) for 10 min and 30 s using an ultrasonic nebulizer (Fisoneb, Karapharm Lab., Marseille, France). FEV₁ was measured before, at 3 min 30 s, and at 10 min 30 s after the beginning of each saline inhalation. At the same time, patients were asked to cough and spit, or whenever they get urged to do so. Saline inhalation was stopped if FEV₁ dropped by 20% from baseline. Sputum samples were processed within 2 h, as follows. Samples were transferred to a Petri dish. Cell plugs were selected from the sample, separated from saliva, weighted and dispersed by the addition of four volumes of 0.1% dithiothreitol (DTT, Sigma Pharmaceuticals, Saint Quentin Fallavier, France) prepared in Dulbecco's phosphate buffered saline (DPBS, Gibco, Invitrogen Corporation, Paisley, UK). After agitation for 15 min at room temperature, DPBS was added and mixed for 15 s. The cell suspension was filtered through a 48 µm nylon mesh to remove mucus and debris. Quality control of the induced sputum was assessed according to the ERS criteria. Filtered sputum were then centrifuged at 400 g for 15 min to separate cells from the fluid phase. Fluid phase was stored at -80 °C until ECP assay.

Cell counts in sputum and serum

Total cell count in sputum was determined using a Thoma haemocytometer and expressed as the number of cells per millilitre of sputum. Cells were resuspended in DPBS at 10⁶ cells/mL, and cytological preparations were prepared by cytocentrifugation (Shandon III cytocentrifuge, Shandon Ltd, Runcorn, Cheshire) of 60 µL of cell suspension at 20 g for 4 min. Slides were stained with May-Grünwald/Giemsa. Differential cell counts were determined on two slides by counting at least 400 nucleated non-squamous cells per slide. The results were expressed as a percentage of total non-squamous cells. Differential cell counts in blood were determined using a Coulter counter and expressed as absolute number of cells.

Eosinophil cationic protein assay in sputum and serum

ECP levels in fluid phase of induced sputum and in serum were determined using a commercially available specific double-antibody radioimmunoassay (Pharmacia Diagnostics, Saint-Quentin, France), as previously described [13]. The intra-assay coefficient of this assay was 5.5% at 9.9 µg/L and 3.5% at 54.7 µg/L. The inter-assay coefficient was 12% at 9.9 µg/L and 17% at 54.7 µg/L.

Statistical analysis

Statistical analysis of the results was carried out using SPSS.11 and STATsoft 5.1 softwares. Analysis of variance for repeated measurements was used to compare Borg score, FEV₁, FEF 25–75, peak expiratory flow (PEF), blood and sputum cell counts and ECP levels before and after exposure to LPS. Differences between groups were analysed using Student's paired *t*-test. Methacholine and allergen (Fel d 1) PD₂₀ were log transformed to fit a normal distribution before analysis. Statistical significance was set at $P < 0.05$.

Results

Patients

Nineteen patients (eight males and 11 females; mean age 26) participated in the study. The characteristics of the patients are summarized in Table 1. Patients sensitized to tree or grass pollen were studied out of the pollen's season. No detectable levels of airborne Fel d 1 were found in the homes of the 19 patients.

Dyspnoea Borg scale, lung function and methacholine bronchial challenge test before pre-exposure to either lipopolysaccharide or saline

Patients did not differ significantly for any of the parameters measured before the two pre-exposures: dyspnoea

Borg scale, PEF, FEV₁ and FEF 25–75 ($P = 0.681, 0.7, 0.83$ and 0.9 , respectively). As well, methacholine PD₂₀ values measured before pre-exposure to saline or LPS did not differ significantly ($675.3 \mu\text{g}$ before pre-exposure to saline as compared with $408.7 \mu\text{g}$ before pre-exposure to LPS, $P = 0.069$) (Table 2).

Effect of pre-exposure to lipopolysaccharide on symptoms and lung function

Inhalation of $2 \mu\text{g}$ LPS did not induce any clinical symptoms, such as dyspnoea and/or fever, in any of the subjects. As well, there was no significant effect of LPS on lung function as assessed during the 6 h that followed exposure: FEV₁ (Table 3), PEF ($P = 0.388$) and FEF 25–75 ($P = 0.325$).

Effect of pre-exposure to lipopolysaccharide on bronchial response to cat allergen

Pre-exposure to $2 \mu\text{g}$ LPS did not significantly modify PD₂₀ Fel d 1 values when compared with values measured after pre-exposure to saline ($P = 0.42$) (Table 2). Indeed, bronchial pre-exposure to LPS was associated with a doubling dose decrease in PD₂₀ Fel d 1 in eight patients, a less than one doubling dose decrease in one patient, and an increase in PD₂₀ Fel d 1 in 10 subjects (four with a doubling dose). A late-phase asthmatic response was observed in four

Table 1. Characteristics of the patients

No.	Age (year)	Sex	Positive skin prick tests to common aeroallergens	Specific IgE to cat allergen (kU/L)	Baseline FEV ₁ (% of predicated value)	Baseline FEF _{25–75} (% of predicated value)
1	23	M	Dpt; Df; cat; grass pollens	1.97	99	72
2	24	F	Dpt; Df; cat	0.98	94	56
3	22	F	Dpt; Df; cat	4.66	96	88
4	24	M	Cat; dog; moulds; tree pollens	3.03	92	84
5	35	F	Dpt; Df; cat; grass pollens	7.12	104	94
6	45	F	Df; cat; dog	5.86	86	63
7	25	M	Dpt; Df; cat	4.08	109	81
8	29	M	Cat; dog	4.60	89	81
9	19	M	Cat; dog	8.45	98	80
10	31	F	Dpt; Df; cat; dog; grass pollens	52.50	110	95
11	28	F	Dpt; cat; grass pollens	35.80	106	105
12	39	M	Cat	1.82	101	82
13	21	F	Cat; moulds; grass pollens	6.41	87	50
14	22	F	Dpt; Df; cat; grass pollens	4.27	105	91
15	21	F	Dpt; Df; cat; grass pollens	30.90	103	115
16	22	M	Cat; dog; moulds; grass pollens	2.53	81	76
17	21	F	Dpt; Df; cat	30.90	108	123
18	24	F	Cat; Grass and tree pollens	0.75	92	84
19	22	M	Dpt; Df; Cat; Grass and tree pollens	0.75	96	69
Mean	26			10.91	97.7	83.6
Median	24			4.60	98	82

Df, *Dermatophagoides farinae*; Dpt, *Dermatophagoides pteronyssinus*; F, female; M, male; FEV₁, forced expiratory volume in 1 s; Ig, immunoglobulin.

Table 2. Methacholine and allergen PD₂₀ values

No.	Age (year)	Sex	Methacholine PD ₂₀ (µg)		Allergen PD ₂₀ (ng Fel d 1)	
			Before saline	Before LPS	Saline pre-exposure	LPS pre-exposure
1	23	M	132	100	25.91	7.7
2	24	F	330	245	11.73	26.97
3	22	F	875	187	20	9.25
4	24	M	250	340	17	22
5	35	F	182	400	16.8	24.2
6	45	F	56	44	4.91	11.7
7	25	M	85	120	9.85	13
8	29	M	570	390	89	42.22
9	19	M	75	30	8	22.75
10	31	F	260	150	0.16	0.04
11	28	F	3600	2560	51.6	42.22
12	39	M	3200	1000	11.84	27.08
13	21	F	31	22.5	5.4	1.33
14	22	F	300	355	52	102.5
15	21	F	125	87.5	10.25	17.94
16	22	M	79	25	71	25.4
17	21	F	400	279	34.7	70
18	24	F	151	230	880.42	270.82
19	22	M	2130	1200	333	118.42
Mean	26		675.3	408.7	87.03	45.03
Median	24		250	230	17	24.2
P-value			0.069		0.42	

LPS, lipopolysaccharide.

Table 3. Mean values of FEV₁ and standard deviations before and during the 6 h that followed exposure to saline or endotoxin

Time	Saline	LPS*
Before exposure	97.41 ± 9.53	97.17 ± 9.15
15 min after	95.64 ± 7.91	94.73 ± 8.15
30 min after	94.95 ± 7.94	93.62 ± 8.78
45 min after	95.95 ± 8.57	93.18 ± 9.35
60 min after	96.43 ± 8.25	93.66 ± 9.56
90 min after	96.27 ± 8.11	94.02 ± 8.64
2 h after	96.05 ± 8.50	94.46 ± 8.68
4 h after	96.23 ± 8.77	95.48 ± 7.36
6 h after	96.00 ± 8.08	94.72 ± 8.24

*P = 0.078, analysis of variance for repeated measurements.

FEV₁, forced expiratory volume in 1 s; LPS, lipopolysaccharide.

subjects in both groups. As well, no significant difference in dyspnoea Borg scale and lung function parameters (FEV₁, PEF, FEF 25–75) was found at the end, and 3, 6 and 24 h after the cat BCT when comparing pre-exposure to LPS with pre-exposure to saline.

Effect of pre-exposure to lipopolysaccharide on cell counts and eosinophil cationic protein levels

No significant changes in eosinophil and neutrophil counts, as well as in ECP levels were observed in blood

within 3, 6 or 24 h that followed cat BCT whatever the pre-exposure, saline or LPS (Fig. 2a). Sixteen subjects produced adequate sputum samples (i.e., 200–400 mg). Allergen bronchial challenge induced no significant changes in eosinophil or neutrophil counts in sputum whatever the pre-exposure (Fig. 2b). In contrast, sputum ECP levels increased significantly after cat BCT, as measured at 24 h. However, this increase was not significantly different when comparing the two types of pre-exposure ($P = 0.082$).

Discussion

In this study, we assessed the effect of low-dose LPS on allergen-induced bronchial and inflammatory responses in allergic asthmatics sensitized to cat allergens. Our data showed that pre-exposure to low-dose LPS did not modify immediate and late bronchial response to cat allergen. It neither changed local and systemic inflammatory cell number, as well as eosinophil activation, as assessed by ECP assay.

The role of endotoxin on specific bronchial responsiveness was previously studied by Boehlecke et al. [15]. These authors pre-exposed mite-sensitized asthmatics to 500 ng/m³ endotoxin for 4 h, taking into account that a ventilation rate of 10 l/min corresponds to a total inhaled dose of 1.2 µg LPS. In the present study, 2 µg LPS was used. This dose may be qualified as low, as it did not

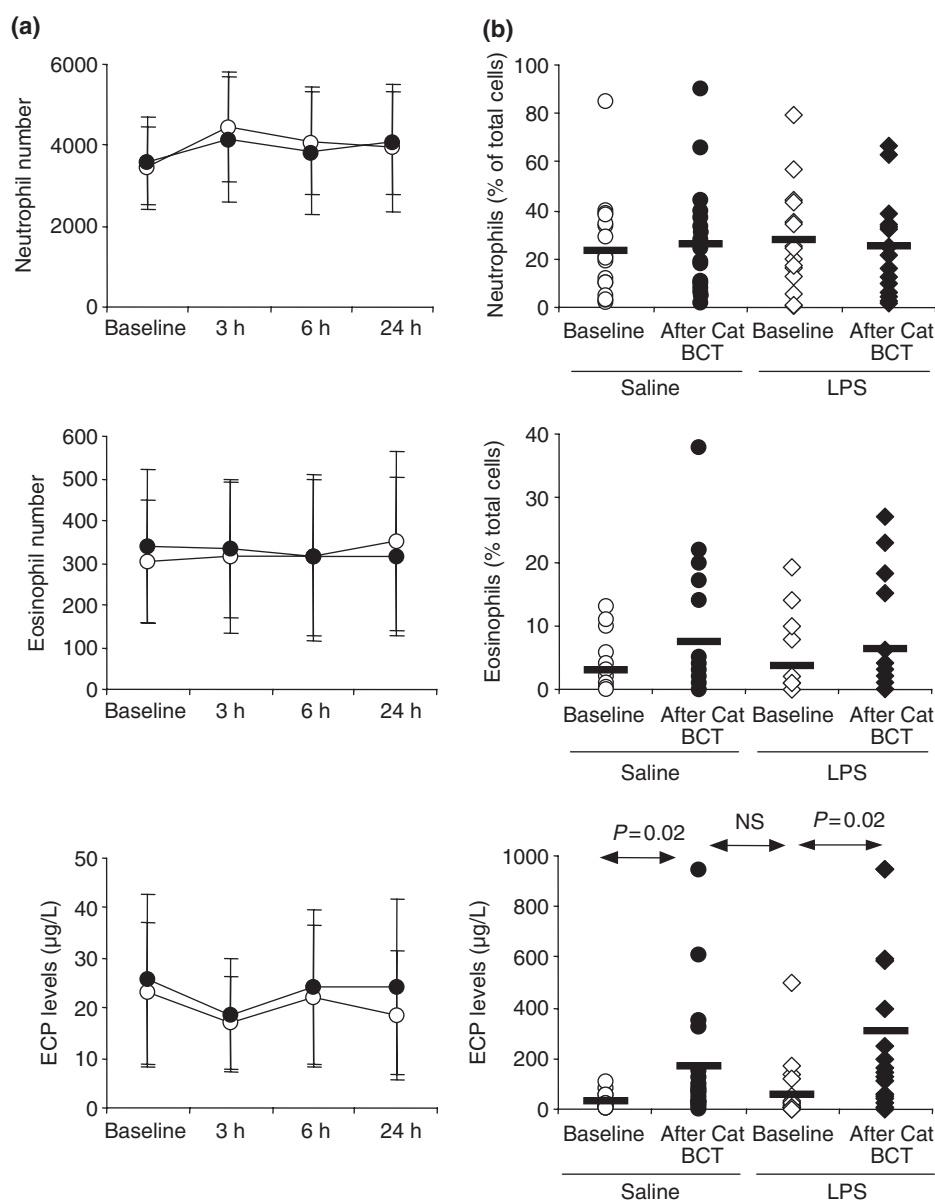


Fig. 2. Cell counts and eosinophil cationic protein (ECP) levels in blood (a) and induced sputum (b) before and after bronchial challenge test to cat allergen (cat BCT). (a) Patients pre-exposed to saline (open symbols) or lipopolysaccharide (LPS; closed symbols). (b) Before pre-exposure to saline or LPS (open symbols) and after cat BCT (closed symbols).

induce any clinical symptoms or changes in lung function. Moreover, it did not result in any airway neutrophil influx as assessed 24 h after exposure. As in the study of Boehlecke *et al.* [15], a single pre-exposure to LPS was used. It was preferred to a repeated exposure, as repeated exposure to endotoxin could induce tolerance, as demonstrated recently by Loh [16]. As well, there are no data demonstrating that repeated exposure for a short period (7–10 days for example) is closer to the natural exposure. In contrast, for allergen, particularly for cat, it has been demonstrated that repeated exposure to low dose induced an increase in bronchial responsiveness

and inflammatory markers, in the absence of clinical symptoms [13, 17].

Whereas Boehlecke *et al.* [15] found that low-dose LPS induced a significant decrease in mite allergen PC₂₀, we found that it did not change cat allergen PC₂₀. Differences in the protocol of exposure could explain this discrepancy. Indeed, whereas in the Boehlecke study patients underwent allergen challenge 1 h after exposure to LPS [15], our patients underwent bronchial challenge to cat allergens 24 h after LPS inhalation because of induced-sputum procedure. As well, in the present study, a dosimeter method, allowing a precise dose delivery, was used to

expose patients to endotoxin rather than an experimental room. Another explanation could be a difference in the particle size of cat allergen and endotoxin used during the nebulizations. Indeed, whereas in the Boehlecke study the mass median aerodynamic diameter of endotoxin particles was 4.9 µm, we chose to nebulize particles with a medium mass aerodynamic diameter of 10 µm. Indeed, we have recently demonstrated that the levels of Fel d 1 that induced immediate bronchial response in asthmatic patients were 20 times lower when the allergen was carried by large particles [18]. According to preliminary data on airborne endotoxin measurements with cascade impactor in swineries, we also nebulized endotoxin on large particles. One can wonder if the number of patients included in our study was sufficient to avoid a possible statistical type II error. However, it was difficult to calculate the number of patients because we had no data about the frequency of non-responsive patients to endotoxin in our population. Our present experimental data are however in agreement with epidemiological ones previously published by Lieutier-Colas et al. [19]. Indeed, these authors did not find any correlation between symptoms and endotoxin exposure in the rat laboratory room. In this study, airborne endotoxin levels were higher than in domestic environments but far lower than in mills or swineries.

Nasal challenge with 1 µg LPS was reported to cause an influx of eosinophils in nasal airways of atopic subjects, suggesting that exposure to low-dose endotoxin may increase allergen-induced bronchial inflammation in asthmatics [20]. Furthermore, in our group, Delayre-Orthez et al. [21] recently investigated the effect of low-level endotoxin on allergen-induced airway inflammation in the mouse and found that a low dose of LPS increased eosinophil number in bronchio-alveolar lavages of sensitized animals, although triggering no neutrophil influx. However, no study addressed so far the effect of pre-exposure to LPS on allergen-induced airway inflammation in asthmatics. In the present study, we found that inhalation of 2 µg LPS did not influence eosinophil counts in blood and sputum, demonstrating that low-dose endotoxin does not exacerbate systemic and local eosinophilic inflammation. Furthermore, we found that pre-exposure to LPS neither triggered eosinophil activation, as assessed by measuring ECP levels.

In conclusion, we have shown that bronchial exposure to low-dose LPS, which may be encountered in domestic environments, had no effect on immediate and late-phase bronchial response, as well as on local and systemic inflammatory response, particularly eosinophils in asthmatics sensitized to cat.

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