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Lipopolysaccharide-induced nasal cytokine response: a dose–response evaluation

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Abstract Information on the dose–response relationship is a prerequisite to defining the non-response threshold of exposure. We investigated whether nasal lipopolysaccharide (LPS) challenges induce an inflammatory response in a dose-dependent way. In three settings nasal lavage was performed before, and 20 min, 1, 6, 23, and 29 h after instillation of 0 μg, 10 μg, and 40 μg LPS for 10 s, in seven healthy subjects. Lavage fluids were analysed for concentrations of interleukin-6 (IL-6), IL-8, tumour necrosis factor-α (TNF-α), histamine, and albumin. Symptoms were recorded by questionnaire and spirometric lung function was assessed after each lavage. The instillation of 40 μg LPS caused a small increase in nasal symptoms. TNF-α was below the detection limit (0.5 pg/ml) in most subjects and, like IL-8 and albumin, showed no relation to the LPS challenge. IL-6 increased over twofold with 10 μg LPS and over 13-fold with 40 μg LPS, with a peak at 6 h after LPS provocation, and the repeated design ANOVA was significant for dose and for time. Six hours after the 40 μg LPS challenge the histamine level significantly increased compared to the saline treatment. We conclude that short-lasting instillation of LPS causes a dose-dependent IL-6 release in the upper airways and minor nasal symptoms.

Keywords Lipopolysaccharides · Nasal lavage · Cytokines · Dose response · Nasal symptoms · Histamine

Introduction

Exposure to endotoxins [defined as lipopolysaccharides (LPS) and other cell wall fragments of gram-negative bacteria] or to pure LPS causes symptoms such as headache, fever, fatigue and malaise, and leads to airway inflammation and increased bronchial responsiveness to methacholine in healthy previously unexposed subjects [12, 20, 23]. The role of the upper airways in modifying incoming air and removing large particles has been well known for many years. It has become apparent that the upper airways must be viewed as both a filter to protect the more distal airways and as a possible site of toxicity. In healthy subjects it has been demonstrated that interleukin-6 (IL-6), IL-1β and tumour necrosis factor-α (TNF-α) increase in nasal lavage fluid a few hours after exposure to swine dust containing endotoxin [30].

Information on the dose–response relationship is a prerequisite for defining the non-response threshold of exposure that should be considered as the safe concentration of endotoxin contamination in airborne dust. Available data on the dose–response relationship are rare concerning the release of pro-inflammatory mediators for LPS provocation. Although Castellan [8] and Rylander [23] have reported a relationship between endotoxin levels and the decrease in the forced expiratory volume after 1 s (FEV_1) in selected normal subjects, the dose–response relationship in a given subject, measured by sensitive local markers of inflammation, has only been reported in the lower airways [15].

The aim of the present study was to evaluate whether albumin and histamine or the pro-inflammatory cytokines IL-6, IL-8 and TNF-α are released in the upper airways after nasal LPS instillation and to evaluate a dose–response relationship. IL-6 is a pro-inflammatory cytokine and can be synthesized by macrophages, lymphocytes, and endothelial and epithelial cells as well as mast cells. It plays an important role in the acute-phase-reaction and in haematopoiesis, and may influence the endocrine and nervous systems [2]. TNF-α is a particularly important mediator of inflammation and cellular immune responses. Over-expression of TNF-α has been implicated in the pathology of severe infection, sepsis and inflammatory tissue destruction 28]. IL-8 is a chemokine and mediates predominantly the migration of neutrophils; it also induces the expression of adhesion molecules. Histamine is one of the hallmarks of the allergic reaction but it is known that histamine can also be released after a second stimulation of basophils by chemokines such as IL-8 [22].

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The inflammatory reaction was assessed in nasal lavage samples taken before, and 20 min, 1, 6, 23, and 29 h after a 10-s nasal instillation of 10 ml pyrogen-free NaCl solution containing 0, 10 or 40 μg LPS of *E. coli* during three experimental sessions in the same subjects.

Materials and methods

Subjects

We looked for non-smoking, non-allergic volunteers. All subjects gave written informed consent, and the study was approved by the local ethics committee. Seven subjects (three females, four males) of 26–43 (median 30) years of age participated in the study. None of the subjects had a history of allergic or non-allergic respiratory diseases and none took any medication at the study time. Their baseline lung function was normal (between 90 and 110% of predicted values). Six subjects had a Phadiatop of class 1 (the Phadiatop system measures IgE levels in response to common allergens). One subject had high IgE levels (Phadiatop class 3) but had never experienced any symptoms of allergy.

Study design

At 1-week intervals, in a single-blind manner, each subject was submitted to a nasal challenge with sterile saline and two doses of LPS. The dosage was partly randomised. Doses of 0 μg LPS and 40 μg LPS were randomised; after a crude data analysis the last dose was chosen to be 10 μg LPS. The 10 μg LPS dose was therefore always given in the last session of the study. In the first session a blood sample for IgE determination was taken.

On each provocation day, the nose was inspected at 09:00 and cleaned with two nasal lavages with isotonic NaCl. The baseline lavage was taken at 09:15, followed by spirometry. At 09.30 a nasal instillation with either 0, 10, or 40 μg LPS was performed for 10 s. The challenge time was identical with the normal lavage sampling time. Lavage samples were taken after 20 min, 1 h, 6 h, 23 h and 29 h , followed by spirometry and assessment of symptoms by a questionnaire.

All provocation sessions were done in the same room at 22 °C and 40% relative humidity. Between nasal lavages the subjects stayed in the same building and they had been asked to keep away from known sources of nasal irritants. The night was spent in their own homes.

All participants completed a symptom questionnaire, with questions about nasal irritation, eye irritation, and throat and airway symptoms. The symptoms were reported using a 10-grade scale from 1, clearly not blocked or no runny nose, to 10, totally blocked or runny nose.

Nasal lavage

The procedure for nasal lavage previously described by Bascom and Pipkorn was used [5, 21]. During lavage, the subject was seated with the neck extended at an angle of approximately 45° and with the soft palate closed. Five millilitres of 0.9% NaCl was instilled into each nostril, using a needleless syringe. After 10 s, the subject flexed the neck forward and expelled the liquid into a plastic basin, which was placed on ice during processing.

The volume of the combined lavage portions was measured and centrifuged at 300 *g* for 10 min. at +4 °C. Aliquots of the supernatant were kept frozen at –80 °C.

Analysis of nasal lavage fluid

The concentrations of IL-6, IL-8 and TNF-α in lavage fluids were measured in duplicate by enzyme-linked immunosorbent assay (ELISA). For IL-6, IL-8 and TNF-α a Quantikine TM high sensitivity, two-site (sandwich) ELISA kit (R&D Systems, Minneapolis, USA) was used. The Quantikine high sensitivity immunoassay uses an enzyme amplification system with alkaline phosphatase. The lower detection limits of the assay for IL-6, IL-8 and TNF-α were 0.156, 31.2, and 0.5 pg/ml., respectively. Absorbency was read at 490 nm using a Microplate Reader Model 3550 (BioRad, Calif., USA) and the results were analysed with Microplate Manager/PC Data Analysis Software, version 4.0. TNF-α was only analysed for 0 and 40 μg LPS challenges. Albumin content was determined using the DC Protein-Assay (Bio-Rad, Calif., USA) according to the manufacturer' instructions, with bovine serum albumin as standard and a detection threshold of 3.8 μg/ml. All samples were analysed in duplicate systematic dilutions. Absorbency was read at 655 nm. Histamine was analysed only for 0 and 40 μg LPS challenges by an ELISA kit (Immunotech, Marseilles, France) with a lower detection limit of 0.5 nmol. Absorbency was read at 405 nm using a Microplate Reader (BioRad, Calif., USA).

Lung function

FEV_1 and forced vital capacity (FVC) were measured according to American Thoracic Society (ATS) criteria with a pneumotachograph Cardiorespiratory Diagnostics System (Med Graphics, St Paul, Minn., USA).

LPS challenges

LPS from *Escherichia coli* serotype 026:B6 extracted by TCA precipitation and gel filtration chromatography, Lot 17H4042 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), was used. For preparation of the LPS instillation solutions, 0, 1 and 4 mg LPS ware added to 1000 ml sterile 0.9% NaCl (Braun AG, Emmenbrücke, Switzerland) resulting in LPS concentrations of 0, 1 μg and 4 μg/ml NaCl which were stored at +4 °C. The provocation solution was allowed to warm up to room temperature for 30 min. Five millilitres of the provocation solution was instilled into each nostril, using a needleless syringe. After 10 s, the subject flexed the neck forward and expelled the liquid into a plastic basin.

IgE determination

The IgE determination was made at an approved medical laboratory (Dr. Violier AG, Zurich, Switzerland) using the Phadiatop FEIA CAP system (Pharmacia & Upjohn, Sweden) according to the manufacturer's instructions. The FEIA CAP system measures IgE levels in response to common allergens. According to Tschopp et al. [29] the sensitivity of the Phadiatop is higher than total serum IgE or skin prick tests, but its specificity is lower than that of skin prick testing. The Phadiatop is therefore well suited for screening purposes.

Statistics

Results are presented as medians with individual values. A two-way analysis of variance with repeated design, taking into consideration the factors dose and time, was performed. Differences between the values after LPS challenge and the basal values (i.e. after saline) were tested by the Wilcoxon signed rank test. Differences were considered significant when the *P* value was less than 0.05. Spirometric lung function parameters were compared using a paired *t*-test.

The dose–response relation was analysed by multiple regression and the dose at which 50% of subjects would react (the LE_{50}) was calculated using the results of the multiple regression analysis (LE_{50} = –constant/dose).

Results

Symptoms

During the placebo session with 0 μg LPS the symptoms increased slightly, indicating that repeated nasal lavage may provoke some irritation. The results of the questionnaire are presented in Table 1. A difference of >2 between the symptom score before provocation and at any point after the LPS provocation is considered to be significant. For eye, throat and airway symptoms no differences between LPS doses was found; however, one subject reported quite strong eye irritation after provocation with 40 μg LPS. Only nose irritation was enhanced in three individuals after 40 μg LPS compared to one person with nose irritation after 0 and 10 μg LPS. The increase in nose irritation was indicated 6 h after provocation. No general symptoms such as shivering, malaise or muscle pain were mentioned.

Lung function

The baseline lung function of all participants was normal (between 90 and 110% of predicted values). The pre-exposure values were lowest at the 10 μg LPS setting and FVC increased over the setting, becoming significant 29 h after exposure compared to the baseline value. No other values changed and no relationship to LPS provocation was found.

Nasal lavage fluid analysis

Of the 10 ml instilled liquid, the mean amount expelled for all subjects and all lavage samples was 7.7 ml (SD 0.4 ml, minimum 6.5 ml and maximum 9 ml).

For IL-6 the analysis of variance was significant for time ($P < 0.01$) and for dose ($P < 0.001$), as it was for the interaction of time and dose. The median IL-6 levels at the different sampling times with the three LPS doses are shown in Fig. 1. The IL-6 values for one subject with the 10 μg LPS challenge were excluded from further analysis because of the high IL-6 level present before LPS instillation, caused by an unknown condition.

The IL-6 release in response to 40 μg LPS is most pronounced 6 h after the challenge and does not return to baseline levels after 29 h. With 10 μg LPS the IL-6 reaction is statistically higher than the placebo treatment only at 29 h (one-tailed).

To assess the effect of the LPS dose, where 50% of the individuals react with an IL-6 release (LE_{50}) a positive response was defined as an increase of more than 3 SD of the group IL-6 values with the placebo challenge (positive reaction Δ > 5.3 ng/ml). For five of the seven subjects ad-

Table 1 Number of subjects grouped by the difference (Δ) between the pre-exposure and post-exposure symptom score during 0 (saline), 10, and 40 μg LPS challenges

Δ Symptom score	Nose *n*			Eyes *n*			Throat *n*			Airways *n*		
	0	10	40	0	10	40	0	10	40	0	10	40
+ (0 or 1)	6	6	4	6	6	6	6	6	6	6	7	7
+ (2 – 5)	1	1	2	1	1	0	1	1	1	1	0	0
+ >6	0	0	1	0	0	1	0	0	0	0	0	0

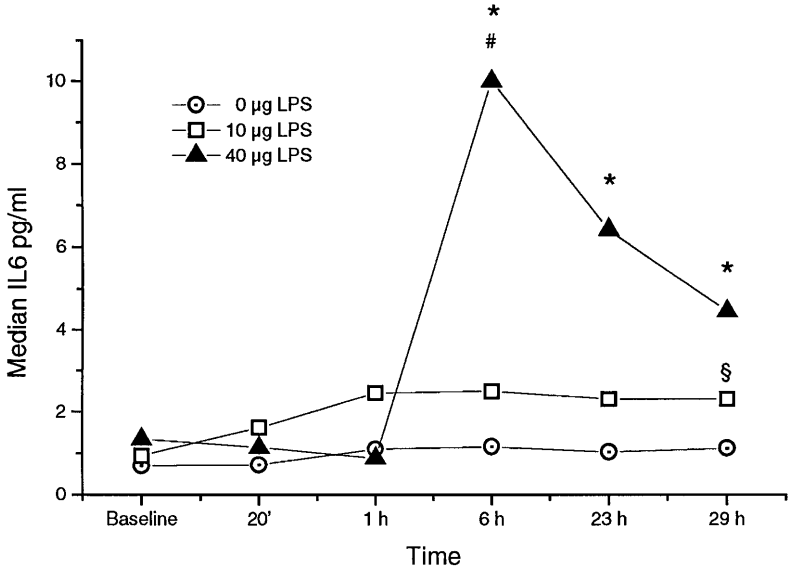

Fig. 1 Interleukin-6 (IL-6) median values after three lipopolysaccharide (LPS) challenges at different times. *$P < 0.05$ from 0 μg curve, #$P < 0.05$ from 10 μg curve, §$P < 0.1$ between 10 μg and 0 μg

Table 2 Number of individuals who showed an interleukin-6 (IL-6) response to the different lipopolysaccharide (LPS) provocation doses

Subjects tested	LPS provocation μg	Reacting subjects	
		n	%
7	0	0	0
6	10	2	33
5	20	3	60
7	40	6	86

ditional IL-6 results with a 20 μg LPS challenge are included. Table 2 lists the number of individuals involved under the different LPS-provocation conditions and the number and percentage which reacted as defined above. The analysis by logistic regression is significant, with a constant estimate of –2.106 (SE 0.883) and a dose estimate of 0.128 (SE 0.048). The LE_{50} calculated is 16.45 μg LPS (SE 6.09).

Figure 2 shows the median curve for histamine with the different LPS challenges. One individual always had histamine levels above the upper detection limit (100 nmol), independent of the LPS challenge. The histamine level increases 6 h after the challenge and is significantly different from the placebo challenge ($P < 0.05$) at this time point.

Only a few TNF-α values lie above the lower detection limit. No effect of LPS could be observed in IL-8 and TNF-α (data not shown). The albumin levels after all LPS challenges increase with time (data not shown). In the analysis of variance, time has a significant influence ($P < 0.01$), but not dose.

Discussion

The study shows an IL-6 release in the upper airways in response to a pure LPS challenge and indicates that in normal subjects the nasal response to LPS is related to the instilled dose. The dose–response relationship can be shown on the basis of how many subjects react to a dose and also on the amount of IL-6 released after the different LPS provocation doses, despite the fact that we did not further analyse the values which exceeded the upper detection limit. It had previously been shown that a nasal instillation of 20 μg LPS caused an IL-6 reaction in 8 (73%) of 11 challenged healthy subjects [31]. This fits very well with our dose–response relationship.

The most important limitation of this study is that the different provocation doses had not been totally randomised. The increase in FVC at the end of the 10 μg LPS setting, which was the last measurement for all participants, may reflect that they wanted to give their best the last time. A learning effect can be excluded; this would have been visible much earlier. The experimental design could also have introduced a bias since each previous LPS instillation, as well as the repeated nasal lavages, could modify the response to the next dose. Nevertheless, the similar baseline values of the parameters measured before the provocation suggests that the subjects had recovered their baseline status. A possible effect of induction of tolerance to LPS on the results is quite impossible since it is known that the early phase of tolerance develops within several hours and lasts no more than 2 days [10]. The subjective symptoms could only minimally serve as a discrimination tool; only three subjects experienced nasal symptoms after exposure to 40 μg LPS and in the session without LPS there was also a slight increase in symptoms.

Wang [30] exposed subjects for 3 h to organic dust containing endotoxins and assessed the cytokine response in the nasal lavage. He found an increase in TNF-α, IL-1β and IL-6 by 7 h after the start of the exposure to 1.2 μg/m^3. The amount of IL-6 released is of the same magnitude as measured here 6 h after instillation of 40 μg LPS. A 3-h exposure to 1.2 μg/m^3 of endotoxin corresponds roughly to an inhaled dose of 3–4 μg endotoxin. The differences in the amount of LPS needed to induce the same effects as endotoxins is well known and not fully understood [9].

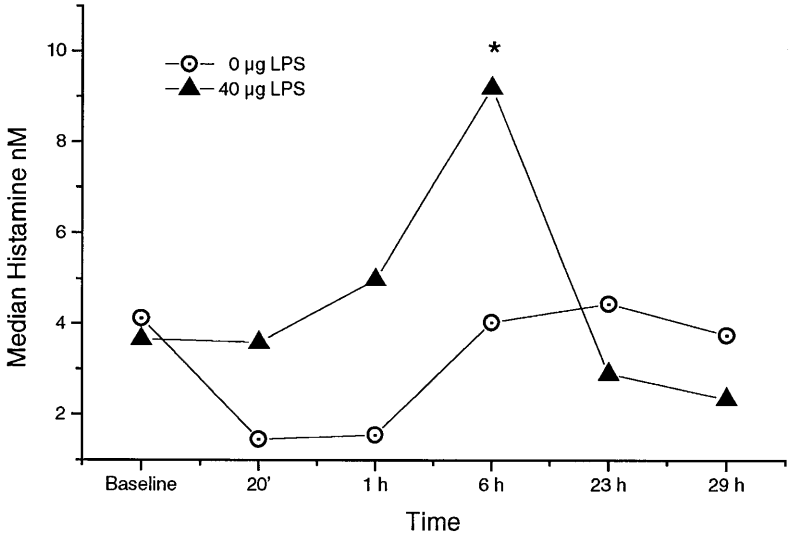

Fig. 2 Histamine median values after two LPS challenges at different times. *$P < 0.05$ from 0 μg curve

TNF-α and IL-8 release were observed after exposure to 10 mg LPS in an experimental design quite similar to the one in the present study [25].

Two explanations for the absence of IL-8 and TNF-α release in this study should be mentioned. First, the reaction took place at a time when no lavage samples were taken; second, the LPS dose used was too low to induce release of TNF-α and IL-8.

After inhalation of 20 μg endotoxin, TNF-α was found in serum as early as 60 min after inhalation and returned to normal after 120 min [15]. It is believed that TNF-α generally precedes the appearance of other inflammatory cytokines [7]. But 7 h after the start of exposure to swine dust TNF-α was still enhanced in nasal lavage, as was IL-6 [30]. After nasal instillation of approximately 10 mg LPS, TNF-α was first increased 6 h after exposure, whereas it was not increased at 3 h [25]. It is therefore not plausible that TNF-α release falls exactly between the two sampling points (1 and 6 h after provocation), especially as the time course of cytokine release shows great inter-individual variability. IL-8 shows a different release pattern. The lavage level of IL-8 increases slowly over time [4], and stays high for some hours, which makes it even less likely that we missed the response.

Michel and co-workers [15] found an increase in TNF-α after inhalation of 50 μg but not after 5 μg LPS in the lower airways. With a dose 250 times higher than we used TNF-α in the nasal lavage increased about four times over baseline [25]. It is most plausible that the instilled amount of pure LPS in our study was not sufficient to induce a TNF-α response in the nose.

This is the first study to have evaluated a histamine response to an LPS challenge in the nose. The histamine level doubled 6 h after the challenge with 40 μg LPS. However, the histamine level after the placebo treatment was increased after 6 h too. Therefore it has to be considered whether repeated nasal lavage could be the cause of the histamine increase. Repeated nasal lavages with physiological saline do not cause an increase in histamine levels [11, 22], whereas challenges with hyperosmolar saline induce a histamine response in allergic subjects [11]. Physiological saline was used and our subjects were not allergic, with the exception of one subject who had raised levels of IgE. The increase in histamine after 6 h could reflect a refilling process of the histamine pool, as Naclerio and co-workers suggested [17]. But the histamine values returned later to baseline level and did not stay high. Data on LPS-induced histamine release in vitro are conflicting. Leal-Berumen and colleagues [13] did not find histamine release in mast cells after stimulation with LPS, but a dose-dependent increase in histamine synthesis in LPS-stimulated macrophages has been demonstrated [27]. In a human nasal mucosa culture system ozone and nitrogen dioxide (LPS was not tested) induced histamine release [28]. Although the histamine response is not proved in the main analyses, our results indicate that there may be an LPS-induced histamine release in the nose. It would have been of interest if the histamine increase had been accompanied by changes in nasal patency measured by rhinomanometry.

Albumin was tested as an effect marker. The albumin level increased significantly over the measured time period with no relation to the LPS dose, although the highest albumin measurements have been found after 40 μg LPS provocation. Albumin as a marker of endothelial permeability could reflect the repeated nasal lavage treatment or vascular permeability having a circadian rhythm pattern, and higher LPS doses are needed to discriminate this from the natural pattern.

Activated mononuclear cells have generally been considered the primary source of IL-6 as well as TNF-α. But macrophages and mast cells are scarce in the nasal mucosa of healthy subjects [6]. However it has been shown that fibroblasts and other tissue structural cells in the nose can produce IL-6, especially in response to LPS [32]. Immunoreactivity for IL-6 was localised in the apical portion of epithelial cells and also the superficial lamina propria [18]. Therefore it is most likely that the cell sources of IL-6 are the epithelial cells, fibroblasts and other structural cells.

It is a common feature that the peak increase in inflammatory mediators in the nose after stimulation with LPS occurs approximately 6 h after stimulation onset [25, 30]. This time course implies that LPS stimulates the production and not the release of pre-formed inflammatory mediators. Different LPS receptors are described; membrane-bound receptors as well as soluble plasma receptors interact with various cell types and show different LPS affinity [19]. Many transcription factors are cell-specific and a number of cytokine-specific transcription factors exist [1]. With the low LPS concentrations and short contact time used in this study, a receptor with very high LPS affinity and good accessibility must be involved which activates predominantly the IL-6 transcription factor. With higher LPS concentrations or longer contact times LPS may bind to receptors with lower affinity and may induce a different cytokine response pattern, e.g. an increase in TNF-α and consequently in IL-8 [3]. Miadonna and colleagues [14] demonstrated enhanced histamine release from human basophils after stimulation with IL-6. Histamine may further down-regulate TNF-α via IL-10 [26]. Whether histamine release is LPS-dose-dependent remains to be tested. It has been shown that IL-8 can induce a histamine response [22]. A dose dependency can be assumed, based on the reasoning above that higher LPS doses will elicit induction of TNF-α and IL-8.

Taken together, nasal mucosa inflammatory response patterns depend not only on the kind of inflammatory stimulus (e.g. LPS, allergen or viral) but also on stimulus intensity.

We conclude that a low-dose and short nasal LPS challenge causes IL-6 release in the upper airways in healthy subjects. The IL-6 release is dose-dependent with regard to the amount of IL-6 released and the number of subjects who react. Our results indicate that LPS may cause histamine release in the nose; however this needs to be investigated in more detail.

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