Inhaled endotoxin in healthy human subjects: A dose-related study on systemic effects and peripheral CD4+ and CD8+ T cells

L.C. Loh a,*, B. Vyas b, V. Kanabar c, D.M. Kemeny b, B.J. O’Connor c

a Department of Medicine, Clinical School, International Medical University, Jalan Rasah, Seremban 70300, Negeri Sembilan, Malaysia
b Department of Immunology, Rayne Institute, Guy’s, King’s and St Thomas’ School of Medicine, London, UK
c Department of Respiratory Medicine and Allergy, Bessemer Rd, Guy’s, King’s and St Thomas’ School of Medicine, London, UK

Received 1 December 2004; accepted 31 May 2005

Summary
Background: Inhaled endotoxin or lipopolysaccharide (LPS) is implicated in the pathogenesis of pulmonary diseases. We investigated the inhalation effects of two different doses of LPS in healthy human subjects.

Methods: Eighteen healthy non-atopic human subjects inhaled either 15 μg (n = 10) or 50 μg (n = 8) Escherichia coli LPS in an open study. As control, each subject had isotonic saline inhalation 1 week before (baseline) and after LPS inhalation. Data collected included those of clinical parameter, induced sputum and peripheral blood CD4+ and CD8+ T cells.

Results: Acute flu-like symptoms and pyrexia were significantly greater in the 50 μg than 15 μg LPS group. Similarly, the increase in sputum and blood total cell and neutrophil counts at 6 h following inhaled LPS were greater in the 50 μg group. Myeloperoxidase, human neutrophil elastase and interleukin-8 in sputum sol, but not blood, showed a trend towards greater increase following 50 μg LPS. All these changes were resolved at one week. In the 50 μg dose group alone, there was a reduction in the proportion of peripheral blood interferon (IFN)-γ-producing CD4+ and CD8+ T cells at 6 h followed by an increase at 1 week after inhaled LPS.

Conclusions: The airway and systemic effects of inhaled LPS are dose-related and predominantly neutrophilic. The changes in the proportions of circulating CD4+ and CD8+ T cells suggests preferential recruitment of IFN-γ-producing T cells into tissue from inhaled 50 μg LPS, followed by reappearance of these cells in blood 1 week later.

© 2005 Elsevier Ltd. All rights reserved.

KEYWORDS
Inhaled endotoxin; Lipopolysaccharide; Sputum; Intracellular flow cytometry; CD4+; CD8+

0954-6111/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rmed.2005.06.003
Introduction

Inhaled bacterial endotoxin can cause lung inflammation\textsuperscript{1–4} and is implicated in the pathogenesis of pulmonary diseases such as organic dust lung diseases\textsuperscript{5,6}, asthma\textsuperscript{7,8}, chronic obstructive pulmonary disease\textsuperscript{9,10} and acute lung injury\textsuperscript{11,12}. Studies using inhaled endotoxin or its pure derivative, lipopolysaccharide (LPS) in human subjects have shown that the nature of inflammation is predominantly neutrophilic\textsuperscript{1,3,4} and dose-related.\textsuperscript{2,13} The latter supports the observation that chronic exposures increase risk for lung injury.\textsuperscript{10,14,15} The threshold for inducing clinical symptoms and lung function changes in healthy human subjects is probably between 30 and 40\textsuperscript{16} mg of inhaled LPS.

To further our understanding of endotoxin-induced lung injury, we investigated\textsuperscript{1} The systemic (clinical effects, airway and blood) effects of inhaled LPS at single doses of 15 and 50\textsuperscript{17} mg, in healthy non-atopic human subjects, up to one week, and\textsuperscript{2} whether there were any changes in the peripheral blood interleukin (IL)-4- and interferon (IFN)-\gamma-producing CD4+ and CD8+ T cells following inhaled LPS at these doses, using the technique of intracellular flow cytometry.

Methods

Subjects

Eighteen healthy human subjects were recruited through local contacts. The inclusion criteria were non-cigarette smokers for at least 6 months and if previously smoked, did not exceed 5 pack years; no physician-diagnosed respiratory disease; non-atopic as defined by negative skin prick reactivity to common aeroallergen, normal spirometry, and methacholine PC\textsubscript{20} of >16 mg/ml. Urine cotinine was measured to confirm the history of non-cigarette smoking.

Study design

This was an open study where 10 subjects [mean (range) age: 31 yr (26–39); five males; mean (95\% CI) FEV\textsubscript{1}: 97 (89–106)\% predicted normal] inhaled 15 \textmu g and eight subjects [age: 28 yr (21–35); five males; FEV\textsubscript{1}: 104 (92–117)\% predicted normal] inhaled 50 \textmu g LPS. After a screening visit to examine eligibility, each subject attended three visits, each separated by 1 week. At each visit, induced sputum and blood were collected at 6 h after inhaling either 0.9\% isotonic saline (visit 1 and 3) or LPS (visit 2) via a breath activated dosimeter (Mefar MB3 dosimeter, Brescia, Italy). This time point for collecting sputum and blood was chosen based on the approximation to the onset of ‘flu-like’ symptoms in healthy human subjects from our preliminary studies of three subjects (data not shown) and findings of neutrophilia at such time point.\textsuperscript{2,17} Temperature, blood pressure, pulse and respiratory rate were recorded prior to and every two hourly (up to 8 h) after inhalation of LPS (visit 2) or saline (visit 3). Spirometry was measured before LPS challenge, during sputum induction and at 8 h after inhalation challenge. Each subject filled in a self-reporting questionnaire on respiratory symptoms and their severity (graded as mild, moderate or severe) at visit 2. The order of inhalation challenges was the same for all subjects and not randomized (Diagram A). The rationale for separating all challenges by 1 week was to avoid conducting serial sputum induction at too close an interval. This might be important because inhaled hypertonic saline used for sputum induction can itself cause airway neutrophilia.\textsuperscript{18,19} Such occurrence might confound the interpretation of sputum results following inhaled LPS challenge. Sputum cellular indices have been shown to be reproducible for up to 1 week.\textsuperscript{20,21} The study protocol was approved by local hospital ethics committee and written informed consent was obtained from all subjects.

Inhalation of LPS

Each LPS solution was freshly made up prior to each inhalation using the powder formulation of \textit{Escherichia coli} serotype 026:B6 (Sigma, Poole, UK) diluted with 0.9\% pyogen-free normal saline, and delivered via dosimeter (Mefar MB3 dosimeter, Brescia, Italy) according to the standard protocol.\textsuperscript{22} Based on a 10\textmu l volume delivered per inhalation, five inhalation of a 0.25 mg/ml LPS solution would
deliver 15 µL LPS, and five inhalation of a 1 mg/ml LPS solution would deliver 50 µL LPS into the airway.

**Sputum induction and processing**

Hypertonic saline at concentrations of 3%, 4% and 5% was inhaled via an ultrasonic nebuliser (DeVilbiss Ultra-Neb 2000, Heston, Wollaston, UK) sequentially for 7 min each. After each 7-min period, subjects were asked to rinse and gargle mouth and throat to get rid of saliva and other debris before coughing into a sterile container. Monitoring of FEV₁ during each inhalation was performed for safety reason. We had intentionally omitted pre-treatment with short-acting inhaled β₂-agonist in order to avoid any possible modifying effect on LPS-induced airway inflammation.

The sputum was selected from saliva and processed within 2 h. Sputum was first homogenised by adding four volumes of freshly made 0.1% dithiothreitol (DTT) (Sputolysin, Calbiochem Ltd, Nottingham, UK) that was then added equal volume of Modified HBSS (without Ca²⁺/Mg²⁺) (Sigma, Poole, UK). The cell suspension was filtered through a 48 µm nylon gauze (BBSH Thompson, Scarborough, Ontario, Canada) and the filtrate centrifuged at 3000 rpm for 4 min at room temperature. The supernatant (sputum sol) was aspirated and stored at −70 °C for future assay while the cell pellet was re-suspended with HBSS. Total cell count and cell viability were determined using trypan blue exclusion. The cell suspension was finally adjusted to 0.5 × 10⁶ cells/ml and placed into cups of Shandon III cytocentrifuge (Shandon, Inc. Pittsburgh, PA, USA) to make cytoslides. After air-dried, cytoslides were stained with Wright’s Giemsa for differential cell count on at least 400 non-squamous cells. These cytoslides were counted by two independent observers.

**Myeloperoxidase (MPO), human neutrophil elastase (HNE) and IL-8 in sputum sol and serum**

MPO and IL-8 levels was measured by radioimmunoassay (Pharmacia & Upjohn Ltd, Milton Keynes, UK) and colorimetric enzyme immunoassay (R&D Systems, Abingdon, UK), respectively, according to standard protocols. Their sensitivity limits were 8 ng/ml and 31.2 pg/ml, respectively. HNE activity was estimated based on a colorimetric reaction assay where the rate at which HNE cleaved a

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Symptoms reported in the (A) 15 µg and (B) 50 µg LPS group.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>Muscle ache</td>
</tr>
<tr>
<td><strong>(A)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6*</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>Subjects</td>
<td>Headache</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>++</td>
</tr>
</tbody>
</table>

Symptoms are in descending order of frequency from left to right columns.
+ mild, ++ moderate, +++ severe.
*This subject also complained of mouth ulcers that lasted for 5 days.
nitroanilide substrate to form a coloured product was measured. Each sample was assayed in singlet and into each well of a 96-well microtitre plate, pipetted in the following order: 100 μl of substrate buffer (7.88 g Tris HCl, 8.77 g NaCl; 400 ml deionised water; adjusted to pH 8.6), 20 μl of study sample, 60 μl of deionised water and neutrophil elastase substrate (20 μl methoxy succinyl-ala-ala-pro-val p-nitroanilide in 25% DMSO). The plate was then immediately placed in a kinetic plate reader (Spectromax 250 spectrophotometer) and shaken for 5 s and read at 405 nm every minute over a period of 30 min at 30 °C. The SoftMax Pro software plotted the progress of each reaction and determined $V_{\text{max}}$ between minimum and maximum optical densities of 0–3 OD units. Individual plots for each sample were scrutinised to see whether the reaction had entered the linear phase and if necessary, sample was further diluted and reassayed. Quality control samples, prepared from samples of disrupted blood neutrophils suspended in hexadecyltrimethylammonium bromide (HTAB) (Sigma, Poole, UK), were placed at the beginning and the end of the sample sequence for each assay. $V_{\text{max}}$ was used to indicate HNE activity, presented as the final result in millioptical density units per minute (mOD/min). The method has the sensitivity limits of 0.1 mOD/min.

Peripheral blood mononuclear cells (PBMC)

Fifty millilitre venous blood was collected from each subject in a syringe containing 1% sodium citrate and processed within 2 h. After dilution 1:4 with Hanks balanced salt solution (HBSS) (Life-Technology, Paisley, UK), PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) at 800g for 20 min at 18 °C and then washed with cold HBSS at 600g for 10 min at 4 °C. Viable cell numbers were determined by trypan blue exclusion then washed twice with HBSS at 200g for 10 min at 4 °C and re-suspended at 1 x 10^6 cells/ml in PBS/ 2% foetal calf serum (FCS) (Harlon-Seralab, Loughborough, UK).

Isolation and purification of CD4+ and CD8+ T cells

CD4+ and CD8+ T cells were isolated by positive selection using anti-CD4 and anti-CD8 magnetic beads and Detach-a-bead antibody (Dynal, Wirral, UK) and re-suspended at 1 x 10^6 cells/ml in culture medium (RPMI medium, supplemented with 5% human albumin serum, 100 IU/ml penicillin/streptomycin, and 2 mmol/l l-glutamine). An aliquot of CD4+ and CD8+ T cells were analysed on a FACScalibur to check purity (Becton Dickinson, Oxford, UK) which was >99% for both CD3+CD4+ and CD3+CD8+ cells.

Cell culture and intracellular cytokine staining

CD4+ and CD8+ T cells at a density of 1 x 10^6/ml were plated out in duplicate into flat-bottom 96-well microtitre plates and stimulated with immobilised anti-CD3 (1:100 dilution) and anti-CD28 (1:1000 dilution) (Pharmingen/Becton Dickinson, Oxford, UK), for 4 days. After 4 days of culture, cells were re-stimulated with PMA (10 ng/ml) and ionomycin (400 ng/ml) (Sigma, Poole, UK) overnight and during the final 6 h, incubated with protein synthesis inhibitor, brefeldin-A (5 μg/ml) (Sigma, Poole, UK). Cell cultures were then gently re-suspended and transferred to FACS tubes and spun down at 200g for 10 min. Supernatants were discarded and the cells washed in 2 ml of PBS/0.1% BSA (Sigma, Poole, UK) and centrifuged at 200g for 10 min.
200 g for a further 10 min. Washed cells were then fixed for 30 min at room temperature in the dark with 1 ml of hypertonic 4% formaldehyde. Cells were centrifuged at 200 g for 5 min and the fixative removed. Fixed cells were then permeabilised by the addition of 2 ml 0.5% saponin solution (Sigma, Poole, UK) for 30 min at room temperature in the dark. These cells were spun down at 200 g for 5 min, and then stained for intracellular cytokines with anti-IL-4-PE and anti-IFN-γ-FITC (Becton Dickinson, Oxford, UK) for 30 min at room temperature in the dark. Cells were washed with saponin solution and fixed with 1% paraformaldehyde, ready for flow cytometric analysis (FACScalibur).

**Data analysis**

All results were expressed as mean and standard error. Significance of difference from baseline, 6 h and 1 week post LPS inhalation was studied using repeated measures analysis of variance (ANOVA). Where significant variation was found, Dunnett’s multiple comparison was used to investigate the significance of change between time points. Since the distribution approximates normality, significance of variance in the airway and systemic effects between subjects receiving 15 and 50 µg LPS was analysed using unpaired t tests. All statistical analysis and graphic representation of data were carried out on the GraphPad Prism™ graphic and statistical package (PC Windows™ version). For all statistical tests, $P < 0.05$ was considered significant.

**Results**

Most subjects experienced flu-like symptoms following LPS inhalation, the commonest of which were headache, chills and muscle ache. These symptoms were acute occurring as early as 2 h and mostly resolved by 24 h. One in the 15 µg group however complained of mouth ulcers that lasted for 5 days. In general, the symptoms were more common and severe in the 50 µg LPS group (Table 1). Temperature was significantly higher in the 50 µg LPS, compared to the 15 µg LPS, beginning from the 4th h onwards (Fig. 1). Spirometric data,

![Figure 2](image-url)  
**Figure 2** Induced sputum total cell and differential counts at baseline, 6 h and 1 week after inhaled 15 µg LPS ($n = 10$) and 50 µg ($n = 6$). **$P < 0.001$, ***$P < 0.01$ compared to inhaled isotonic saline (baseline visit 1). Bars represent mean and standard error.
blood pressure, pulse and respiratory rate, were not significantly altered at either dose (data not shown).

Total cell and neutrophil counts in induced sputum were significantly increased at 6 h following LPS inhalation, compared to inhaled saline (baseline visit 1) with a greater trend towards increase in the 50 µg, compared with the 15 µg dose [mean increase (SEM): 22.36 (3.09) vs. 15.17 (4.27) x 10^6 cells/ml of total cells; P = 0.25; 19.71 (2.54) vs. 13.82 (3.80) x 10^6 cells/ml of neutrophils; P = 0.29]. By 1 week, sputum total cell and neutrophil counts had returned to baseline in both groups. The levels of sputum macrophages, lymphocytes and eosinophils were not significantly altered by LPS inhalation at either dose (Fig. 2).

In peripheral blood, total white cell and neutrophil counts were also significantly elevated at 6 h following LPS inhalation, compared to inhaled saline (baseline visit 1). The increase was significantly greater with 50 µg than with 15 µg LPS [mean increase (SEM): 7.59 (0.91) vs. 4.58 (0.82) x 10^6 cells/ml of blood neutrophils; P = 0.007]. By 1 week, these blood changes had returned to baseline in both groups. There was a small but significant reduction of blood monocytes at 6 h following 50 µg, but not 15 µg inhaled LPS, while lymphocytes and eosinophils remained unaltered (Fig. 3).

Overall, inhalation of LPS increased sputum sol MPO, HNE and IL-8 at 6 h, compared with inhaled saline (baseline visit 1). The increase of MPO and HNE was statistically significant at 15 µg LP, while the increase in IL-8 was statistically significantly at 50 µg LPS. By 1 week, their levels were comparable to those at baseline (Fig. 4). Except for a minimal trend towards increase in serum MPO following inhaled LPS compared to inhaled saline (baseline visit 1), the HNE activity and IL-8 levels (except two) in all serum samples were below quantifiable levels [data not shown].

At 50 µg dose alone, there was a reduction in the proportion of peripheral blood IFN-γ-producing...
CD4+ [mean (SEM) %: 3.59 (0.67) vs. 1.60 (0.22)] and CD8+ T cells [5.99 (0.57) vs. 2.84 (0.65)] at 6 h following inhaled LPS (CD4+: 6.41 (1.03); CD8+: 10.86 (2.46)]. The increase at 1 week later was statistically significant when compared with baseline (in CD4+ cells) and 6 h following inhaled LPS (in CD4+ and CD8+ cells). Otherwise, there was no significant difference in the proportion of circulating IL-4-producing CD4+ or CD8+ cells at either dose of LPS (Figs. 5 and 6).

Discussion

We have shown that the systemic effects of inhaled LPS were greater at 50 µg than at 15 µg dose in healthy human subjects. The inflammatory nature was predominantly neutrophilic and more prominent in airway than in blood. It was transient in that they were not present after 1 week. Most unexpected finding was a reduction in the proportion of circulating IFN-γ-producing CD4+ and CD8+ T cells at 6 h after inhaled LPS, followed by a significant increase in these cells 1 week later. This pattern of change was only observed at 50 µg dose.

The pyrexia and acute ‘flu-like’ symptoms reported in our findings are similar to those from other studies, but perhaps more prominent. Although a 30–40 µg dose is probably the threshold for inducing symptoms and lung function changes in healthy subjects, we showed that most subjects receiving 15 µg dose were symptomatic. Two subjects from the group graded the symptoms as severe and one of them had mouth ulcers that lasted for 5 days. Despite the symptoms, we did not report any impairment of FEV1 or FVC in both groups. Others have shown both a reduction of FEV1 and FVC in healthy subjects challenged with inhaled LPS. Kline et al. recently showed that the provocation concentration of inhaled LPS to induce a 20% fall in FEV1, varied considerably in 72 healthy subjects. In those who were sensitive to the effects of inhaled LPS, their peripheral blood monocytes elaborated less IL-6 and IL-8, suggesting that there are inherent differences in individual responsiveness to inhaled LPS.

Like previously reported, a single inhaled LPS in healthy subjects evokes a predominantly neutrophilic response. Michel et al. showed a dose–response relationship between airway and blood inflammatory responses based on weekly bronchial challenges with ascending doses of inhaled LPS in healthy subjects. Our findings add to the evidence of dose-related effects and further extend our understanding on this in showing that the effects of single inhaled LPS at these studied doses is transient in healthy subjects, as evidenced by the absence of airway neutrophilia after 1 week of LPS challenge. This is important because activated neutrophils have been shown to perpetuate further neutrophil recruitment, by a positive autocrine
feedback loop, independent of initiating stimulus of the inflammatory response in disease state such as bronchiectasis. This process is possibly mediated by IL-8, a potent neutrophil chemoattractant, the degree of which is both amplified and sustained in the presence of IL-1β and TNF-α. This view may partly explain the difficulty in correlating microbiologic findings and inflammation during the clinical episodes of infection.

It is also noteworthy that although the neutrophil-related mediators and IL-8 showed towards a greater increasing trend following inhaled 50 µg compared to 15 µg LPS dose, it was not consistent. This lack of parallel increase with neutrophilia suggests that the LPS-induced response at these studied doses is primarily one of cell recruitment, not enhanced activation/degranulation. This concept is also supported by others in their findings of normal or reduced ratios of neutrophil mediators over neutrophil number.

It is well established that bacterial endotoxin induces a Type 1 T cell response in human subjects. LPS is capable of stimulating human T cells directly but this process requires cell-to-cell contact with accessory cells such as monocytes and is MHC unrestricted. Our novel observation of a trend towards reduction in the proportion of blood IFN-γ-producing CD4+ and CD8+ T cells 6 h after 50 µg inhaled LPS is best explained by preferential recruitment of these circulating cells to the local sites of inflammation or lymphoid tissue. The observation of significant increase in the proportion of blood IFN-γ-producing CD4+ and CD8+ T cells after 1 week was unexpected, and may be explained as a ‘rebound’ phenomenon in that the earlier recruited IFN-γ-producing T cells re-emerge in peripheral circulation. In animals, LPS injection is shown to cause an accumulation of IFN-γ-producing T cells in pleural cavity that resolves after 96 h, suggesting that the recruitment process is temporary after a single challenge. These T cells may be confined to memory cells alone. Further research is required to define the phenotypes of these T cells at 1 week after inhaled LPS and to study whether 50 µg dose represents a threshold level of enrolling these T cells.
Inhaled endotoxin in healthy human subjects

Figure 6 A representative FACS dot plot from a subject showing a reduction in the proportion of IFN-γ-producing CD4+ and CD8+ T cells at 6 h (visit 2) followed by an increase at 1 week after inhaled 50 μg LPS (visit 3).
Acknowledgements

The authors wish to thank Dr. Paul Linacre and Ms. Jo Thompson from GlaxoWellcome R&D for the technical support in the measurement of sputum sol and serum MPO, HNE and IL-8.

References