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Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid

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ABSTRACT: Bacterial endotoxin has been suggested as responsible for the development of subjective symptoms and transient or chronic lung function impairment seen after exposure to organic dusts in cotton mills, poultry houses, swine confinement buildings and saw mills. Animal experiments have demonstrated bronchoalveolar neutrophilia being the most prominent cell response in animals following bacterial lipopolysaccharide (LPS) inhalation. The present study was conducted to obtain information on some aspects of the early inflammatory response to inhaled LPS in man.

Eight healthy nonsmoking subjects, 23–27 yrs old, underwent bronchoalveolar lavage (BAL), 3 h after a provocation test with 100 μg LPS from *E. coli* dissolved in 2 ml isotonic NaCl. The solution was aerosolized with a jet nebulizer and inhaled. The calculated dose delivered to the lung was approximately 25 μg, which equals exposure in some occupational settings. The BAL data for each individual subject were compared with data from a control BAL performed at least 6 weeks prior to the LPS challenge.

The major cellular response to LPS, reflected in BAL fluid, was an approximately hundredfold increase in neutrophils. The total number of lymphocytes was on average tripled. The alveolar macrophage phagocytosis of opsonized yeast particles *in vitro* was significantly reduced. A further indicator of an ongoing inflammation was an increase in fibronectin. No changes were seen in the levels of BAL albumin, indicating that the elevated level of fibronectin could not be explained by an increased permeability, but rather by a local production. The results correspond with data from animal studies and further supports the hypothesis that bacterial LPS is important in the pulmonary reaction induced by organic dusts.

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Inhalation of organic dusts in such places as cotton mills, poultry houses, swine confinement buildings and saw mills has been associated with the development of subjective symptoms and transient or chronic lung function impairment [1, 2]. These dusts are contaminated by endotoxins from the cell walls of Gram-negative bacteria (lipopolysaccharides (LPS)), which have been suggested to be the major causative agents of the reactions observed in exposed humans [3–5]. In experimental studies in man, inhaled LPS has been found to induce fever and chills, accompanied by transient bronchoconstriction, bronchial hyperreactivity and a decrease in carbon monoxide transfer factor [6, 7].

It is now realized that most symptoms occurring after exposure to cotton and other organic dusts are related to acute or chronic inflammation induced by cell activation and inflammatory mediator production. In view of this, the possible role of LPS in the inflammatory response should be investigated. Inhalation studies with LPS in humans have primarily evaluated clinical responses. No information is available on cell responses or mediators after inhalation. Although, an abundance of data exists on effects concerning pulmonary pathology after *i.v.* injection [8–11], this exposure route does not correspond to environmental exposure and the information could be limited in usefulness for environmental risk assessments.

According to experience from animal inhalation models, neutrophil migration from the blood stream to the epithelium and air spaces is the most pronounced cell reaction [12, 13]. There are several observations which indicate that activated alveolar macrophages

(AM) are at least partly responsible for this neutrophil recruitment [13, 14].

The present study was performed to obtain information on the inflammatory cell response to inhaled LPS in man. The exposure concentration of LPS used was based on measurements of LPS concentrations in cotton cardrooms and the amounts used in recent inhalation studies in man [4, 6, 7].

Subjects and methods

The subjects were eight healthy students, 23–27 yrs of age (mean 25 yrs). None had a history of asthma or previous exposure to LPS. Three subjects had a history of mild allergic rhinoconjunctivitis to tree or grass pollen. The study was conducted outside the allergy season, during wintertime. All were free of noticeable airway infection during a period of at least six weeks prior to the start of the study. The subjects were informed of the possible occurrence of symptoms following exposure, as described below. The study was approved by the local Ethics Committee.

All subjects were investigated with bronchoalveolar lavage (BAL) at least six weeks before the LPS exposure and three hours after the onset of exposure.

LPS exposure was performed using purified LPS from *Escherichia coli* (*E. coli* 026, B6, Sigma Chemical, St. Louis, USA), of which 100 μg was suspended in 2 ml isotonic NaCl and inhaled for a period of 10–15 min with a Pari Boy inhaler. Preceding experiments have shown the equipment to produce particles with a mass median diameter of 3.5 μm (range 0.5–5.5 μm). The delivered dose has been calculated to be approximately 50% [6].

The method of flexible fibreoptic bronchoscopy with BAL and the processing and staining of the BAL fluid cells were performed as outlined previously [15]. In short, BAL was conducted under local anaesthesia, with lidocaine together with atropine as the only premedication. Four boluses of 60 ml phosphate buffered saline (PBS) at 37°C were infused into the right middle lobe and gently suctioned back to a siliconized vessel placed in ice water. The fluid was chilled during all processing steps. Cytocentrifugal specimens were stained with May-Grünwald-Giemsa for standard differential counts, and acid toluidine blue counterstained with Mayer's acid haematoxylin for mast cells and lysozyme-R antibody (Dakopats) and immunoperoxidase technique for lysozyme positive macrophages.

A questionnaire was used to assess the presence of subjective symptoms following the pre-exposure BAL and the LPS inhalation followed by the post-exposure BAL. In this, the subject marked the time period after exposure when they had experienced airway irritation, chest fitness, chills or febrile reactions, muscle pain, headache or other symptoms.

Alveolar macrophage phagocytosis was measured as percentage of engulfment positive cells, using a glass surface adherence method as described previously [16]. The method has been modified for the use of bronchoalveolar lavage cells. Briefly, 200,000 cells in 200 μl medium containing 10% pooled human AB+ serum were allowed to adhere to glass surface for 30 min in cell culture conditions. After rinsing the non-adherent cells away, yeast cells labelled with fluorescein isothiocyanate (FITC) and opsonized with human serum were added to the slides (2.5×10^7 yeast cells in PBS buffer solution, pH 7.4). After 30 min the phagocytosis was stopped by dipping the slides in ice-cold PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA). The fluorescence of non-ingested yeast cells was quenched by dripping toluidine blue in saline (1 $mg \cdot ml^{-1}$, pH 4.7) onto the slides. Yeast cell adherence (attachment) to an AM was defined as visible contact between a brown yeast cell and an AM. Engulfment was defined as the presence of fluorescent yeast cells within an AM. Fifty consecutive alveolar macrophages were counted. The results were expressed as total numbers of yeast cells engulfed and as percentage of cells that had engulfed one or several yeast particles.

Albumin was measured using the rocket electrophoresis technique [17].

Fibronectin was analysed with a double-sandwich enzyme-linked immunosorbent assay (ELISA) technique. Microtitre plates (Nunc, Denmark) were coated with rabbit-antihuman fibronectin antibodies (Dakopatts, Denmark), diluted 1:2,000 in carbonate buffer pH 9.6. After incubation at room temperature for 24 h the titre plates were carefully washed. PBS containing 0.05% albumin was added for 1 h and then washed away. BAL fluid sample was added in a diluted series together with horse-radish peroxidase-labelled antihuman fibronectin 1:2,000 (Dakopatts) as second antibody and the plates were incubated for 90 min. The amount of bound peroxidase, which is proportional to the amount of fibronectin in the sample, was measured by analysing the enzymatic activity on ortho-phenylenediamine. Plasma fibronectin of nephelometric quality was provided from Sigma Chemicals and used as standard. The detection limit was 10 $\mu g \cdot l^{-1}$. Intra- and interassay variation was below 7%.

Statistics

Wilcoxon's nonparametric signed rank test for paired observations was used. A p-value <0.05 was considered significant.

Results

The subjects tolerated the BAL procedure well. In the initial lavage, before the endotoxin exposure, no adverse reactions were reported and none of the subjects had fever.

After the exposure to endotoxin, three of the eight subjects reported chest tightness and airway irritation 2–4 h after exposure. Five subjects experienced febrile reactions with shivering beginning 4.5–6 h after the

exposure and lasting for 2–3 h. Muscle pain was reported by one subject 5–8 h after exposure. Headache was reported by two subjects during the same time period. All subjects recovered completely within the following hours without any persisting symptoms.

The median amount of BAL fluid recovered after the reference BAL, before exposure, was 66% of the instilled volume (interquartile range 55–73%), which was not significantly changed after exposure. The total number of cells is reported in table 1.

The total number of cells increased significantly, primarily owing to a pronounced, approximately hundredfold, increase in the number of neutrophils. The number of lymphocytes was also significantly increased.

An increase in the number of macrophages was seen, although this did not reach statistical significance. The proportion of lysozyme positive macrophages decreased somewhat, and the number of mast cells was unaffected by the exposure.

Table 2 reports phagocytosis by alveolar macrophages. It is seen that there was a slight decrease in phagocytosing capacity, but that the number of yeast adherence cells increased.

Data on proteins in BAL fluid are reported in table 3. The amount of total protein increased significantly, whereas albumin was not affected by the exposure. The amount of fibronectin was almost doubled, although the range between values was relatively large.

Table 1. – Data on cell content in BAL fluid before and 3 h after LPS inhalation

	Total cells $\times 10^7 \cdot l^{-1}$	Total neutrophils $\times 10^7 \cdot l^{-1}$	Total lymphocytes $\times 10^7 \cdot l^{-1}$	Total macrophages $\times 10^7 \cdot l^{-1}$	Lysozyme positive macrophages % of macrophages	Total mast cells $\times 10^4 \cdot l^{-1}$
Before exposure*						
Median	6.8	0.06	0.49	5.9	7.5	0.06
Interquartile range	5.3–13.7	0.02–0.10	0.30–1.14	4.85–13.1	6.5–12.5	0–0.20
After LPS exposure						
Median	21.6	8.7	1.67	10.1	5.0	0.29
Interquartile range	15.5–28.9	2.5–14.9	0.10–2.46	8.1–14.1	4.0–5.5	0.10–1.42
	p<0.01	p<0.001	p<0.05	NS	p<0.02	NS

*: BAL before exposure was performed at least 6 weeks prior to LPS exposure; Statistical analysis by Wilcoxon's paired rank sum test; BAL: bronchoalveolar lavage; LPS: lipopolysaccharide; NS: not significant.

Table 2. – *In vitro* phagocytosis of opsonized yeast particles by alveolar macrophages before and 3 h after LPS inhalation

	Total number of engulfed yeast particles·200 cells^{-1}	Yeast engulfment positive cells %	Yeast adherent cells %
Before exposure			
Median	272	92	3
Interquartile range	244–304	91–94	1–4
After LPS exposure			
Median	204	76	7
Interquartile range	102–238	60–79	5–13
	p<0.05	p<0.02	p<0.05

Statistical analysis by Wilcoxon's paired rank sum test.

Table 3. – Data on proteins in BAL fluid before and 3 h after LPS inhalation

	Total protein $mg \cdot l^{-1}$	Albumin $mg \cdot l^{-1}$	Fibronectin $\mu g \cdot l^{-1}$
Before exposure			
Median	66	36	122
Interquartile range	60–79	28–55	78–140
After LPS exposure			
Median	82	36	212
Interquartile range	74–100	33–52	86–350
	p<0.05	NS	p<0.05

Statistical analysis by Wilcoxon's paired rank sum tests. For abbreviations see legend to table 1.

Discussion

The inhaled dose of LPS in the present experiments can be estimated to be about 50 μg. With a 50% deposition at this particle size range, the pulmonary dose would be 25 μg. This can be compared to levels encountered in such occupational settings as cotton mills, swine confinement buildings and poultry houses [2]. In these environments, levels of about 1 $\mu g \cdot m^{-3}$ have been measured using the Limulus lysate technique. As the endotoxin in those environments is chiefly bound to bacterial cells, the actual content of endotoxin is not reflected by the Limulus value. Using chemical methods, the amount has been estimated to be about 10 times greater [6]. This would result in a daily exposure (6 $m^3 \cdot day^{-1}$) to about 60 μg endotoxin and a dose of about 30 μg estimating the deposition of this particle size range to be about 50%.

The clinical symptoms reported by the subjects in the study are similar to those previously reported in inhalation studies on endotoxin [7] and those reported by workers with symptoms of toxic alveolitis (TA) or the organic dust toxic syndrome (ODTS) [2]. Although the febrile reactions with muscle pain and headache were quite pronounced, all subjects had completely recovered within a few hours, without any persisting symptoms. The reason why the majority of our subjects suffered from a febrile reaction, compared with only a few in a preceding study with similar dose levels [6], is unclear. Late onset fever may occur after a standard BAL procedure, with an incidence of 1–5% at different centres (3% at our laboratory). It is possible that the BAL procedure itself in some way influenced the LPS-induced inflammation and, thereby, increased the tendency to develop fever.

The major cellular response after LPS exposure was a marked increase in the total number of neutrophils. This agrees with findings from animal models with inhalation of LPS or organic dusts, as well as observations in cotton workers experimentally exposed to cotton dust [4, 5].

It has recently been demonstrated in an animal model that the influx of neutrophils into the airways follows a much earlier migration of neutrophils into the lung interstitium [14]. This early phase of migration into the lungs corresponds to the time sequence for secretion of mediators from alveolar macrophages [18, 19].

In addition to the increase in neutrophils, the number of lymphocytes increased in the lung lavage. This is consistent with previous observations in animals. The data from such experiments suggest that the number of lymphocytes remains elevated for up to 74 h after exposure, whereas neutrophils reach a peak at 24 h. The continued presence of an elevated number of lymphocytes in the lung could reflect a stage or priming and that subsequent exposures to LPS or other irritating agents could cause more pronounced effects.

A more than twofold increase of fibronectin (FN) was observed in the LPS-exposed group compared to the controls. FN is an extracellular matrix glucoprotein with binding sites for several connective tissue matrix components, *e.g.* collagen, fibrin and heparin. FN has a chemotactic effect on fibroblasts [20], as well as on monocytes [21]. It also facilitates monocyte adherence and acts as a non-immune opsonin, upregulating the phagocytic function in neutrophils [22] and monocytes [23, 24].

The mechanisms behind the increased FN concentration in the present study are unclear. As the increase was already present 4 h after exposure, a more complex network of cytokine-cell responses is less likely to have occurred. It is, therefore, reasonable to assume that LPS, either directly or mediated by a first line cytokine response, induced the synthesis and/or secretion of FN.

One possible source of FN could be passive leakage from blood through the lung interstitium, due to an increased vascular permeability. However, the concentration of albumin in BAL fluid was unchanged throughout the study, which makes such explanation less conceivable. Macrophages activated by gamma-interferon, derived from activated lymphocytes, are also known to secrete FN [25]. The finding of increased numbers of BAL lymphocytes gives some, but fairly weak, support to such mechanisms being involved.

The major target for LPS is probably the macrophages. LPS induces macrophage secretion of neutrophil chemotactic and neutrophil activating factors [26]. The neutrophils may themselves be a source of FN [27] as well as influencing endothelial cells to produce this glucoprotein [28]. The early and pronounced increase of neutrophils in the present study also supports the idea that the neutrophils are strongly related to the increase of FN. The likelihood for the neutrophils and not the macrophages to be a major source of FN is further supported by the knowledge that LPS is known to suppress the macrophage secretion of FN [29], possibly due to the release of prostaglandin E_2 (PGE_2) [30].

The data on phagocytosis by alveolar macrophages was unexpected. It has previously been reported, using a guinea-pig model, that phagocytosis is increased after exposure to LPS [31]. On the other hand, other data demonstrate that there is an initial decrease in the number of macrophages recoverable by lavage during the first hours after an exposure to LPS [32]. This finding reflects the increased activity of macrophages with an increased adherence to the alveolar cell wall during the early phases of activation and mediator secretion. It is postulated that had the BAL been performed at 24 h after exposure, the phagocytosing capacity of macrophages would have been increased.

In conclusion, the bronchoalveolar cellular response to inhaled LPS in humans causes a pronounced neutrophil increase at 4 h, which corresponds to data from animal studies. A further indicator of active inflammation is the increased amount of fibronectin in the lavage fluid. The results support the hypothesis that the contamination by bacterial LPS in organic

dusts contributes to the inflammation seen after inhalation of such dusts and probably also to the acute and chronic clinical disease.

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