The potential of various lipopolysaccharides to release monocyte chemotactic activity from lung epithelial cells and fibroblasts


ABSTRACT: Although the cytotoxicity of lipopolysaccharide (LPS) derived from Pseudomonas aeruginosa, i.e. Limulus amoebocyte lysate activity, is less potent than that from Escherichia coli 0127:B8, P. aeruginosa induces prominent sustained lung inflammation, as in cystic fibrosis. The present study was designed to examine the potential for several LPSs obtained from E. coli and P. aeruginosa to release monocyte chemotactic activity (MCA) from lung cells.

LPSs differentially stimulated A549 cells, BEAS-2B cells and lung fibroblasts to release MCA (P. aeruginosa > E. coli 0127:B8 from Difco >055:BS from Sigma >026:B6 (Sigma)). E. coli 0127:B8 (Sigma) and 0111:B4 (Sigma) did not stimulate these cells. MCA was determined by means of checkerboard analysis. Molecular sieve column chromatography revealed four chemotactic peaks. The release of MCA was inhibited by cycloheximide and lipoxygenase inhibitors. Experiments with blocking antibodies suggested that much of the MCA was secondary to monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Thus, the concentrations of these chemoattractants were examined and it was found that the potency of the various LPSs to stimulate MCA closely paralleled their potency in releasing MCP-1 and GM-CSF. Serum augmented the release of MCP-1 and GM-CSF. However, the differences among LPSs from E. coli and P. aeruginosa in stimulating A549 cells were observed.

These data suggest that Pseudomonas aeruginosa lipopolysaccharide may stimulate lung cells to release more monocyte chemotactic activity than lipopolysaccharides derived from Escherichia coli, leading to sustained prominent lung inflammation.


Lipopolysaccharide (LPS) is a potent secretagogue for a variety of cytokines from resident and inflammatory cells. Chemical isolation of lipid A and its water-soluble form (triethylammonium salt) confirmed that lipid A was the active domain responsible for the induction of pathophysiological LPS effects [1–3]. The results of a great number of biological experiments show that for full expression of typical in vivo manifestations of LPS, such as fever and hypotension, the simultaneous presence of a bisphosphorylated 1–6-linked α-glucosaminoglycan disaccharide carrying six acyl groups, four molecules of (R)-3-hydroxy fatty acid (C10–C16) and two secondary fatty acids in the form of two (R)-3-acetoxyxynyl groups in a defined structural arrangement (as in Escherichia coli) is a prerequisite [4].

However, the lipid A structure of some nonenterobacterial LPSs has been found to differ from Haemophilus and enterobacterial species in several parameters. For example, in the lipid A of Chromobacterium violaceum [5, 6], Neisseria meningitidis [7], Pseudomonas aeruginosa [8], and Bacteroides fragilis [9], the nature, number, chain length and location of fatty acids are different. In contrast to the asymmetric acylation pattern of fatty acids attached to the N-glucose disaccharide in the lipid A of Haemophilus and E. coli, a symmetric distribution is present in C. violaceum and N. meningitidis lipid A [5, 6]. The 3-hydroxy fatty acid chain lengths are smaller in the lipid A of P. aeruginosa than Haemophilus and enterobacterial lipid A. The major species of P. aeruginosa lipid A contains only five fatty acids, as the primary acyl residue at position 3 of the reducing N-glucose residue is lacking [8]. Thus, the LPS of P. aeruginosa is significantly less toxic than enterobacterial LPS, and the presence of only five fatty acyl residues in lipid A may account for its low toxicity [8, 10].

The interaction of LPS with cells of mononuclear system is perhaps the central event that triggers systemic reactions, resulting in endotoxic effects [11]. LPS activates mononuclear phagocytes both in vivo and in vitro to enhance functional capacity. Thus, both the cytotoxic activity and phagocytic capacity of macrophages are significantly enhanced on exposure to biologically-active LPS [12, 13]. Conversely, LPS induces the production of inflammatory mediators and immunoregulatory cytokines, including prostaglandins, leukotrienes, platelet-activating factor, superoxide anion, hydrogen peroxide, nitric oxide and interleukins as well as tumour necrosis factor-α [14]. These secondary hormone-like mediators possess potent intrinsic bioactivity and contribute to the overall manifestation of endotoxic effects [14]. In this sense, the LPS molecule is not itself toxic. As recognized by THOMAS...
[15], it is the response of the host organism to LPS that makes LPS "poisonous". This seems to be true for cellular systems, including endothelial cells, fibroblasts and epithelial cells.

Although the cytotoxicity of LPS derived from *P. aeruginosa*, i.e. Limulus amoebocyte lysate (LAL) activity is less potent than that from *E. coli* 0127:B8, *P. aeruginosa* infection induces a sustained prominent inflammation in the lung, including in cystic fibrosis, diffuse panbronchiolitis and chronic inflammatory lung diseases. In the present study, experiments were carried out to clarify the endotoxic effects of various LPSs in terms of the release of monocyte chemotactic activity (MCA) from type II alveolar epithelial-like cells. A549 cells, a bronchial epithelial cell line, BEAS-2B cells and lung fibroblasts were exposed to A549 cells, BEAS-2B cells, and human tissue culture dishes. After 4±6 days in culture, the cells reached confluence and were then used for experiments.

### Methods

**Culture and identification of type II alveolar epithelial cells, human foetal lung fibroblasts and BEAS-2B cells**

Because of difficulty in obtaining primary human type II epithelial cells of sufficient purity, A549 cells (American Type Culture Collection (ATCC), Rockville, MD, USA), an alveolar type II cell line derived from an individual with alveolar carcinoma [16], were used. These cells retained many of the characteristics of normal type II cells such as surfactant protein, cytoplasmic multilamellar inclusion bodies and cuboidal appearance, and had been extensively used to test type II pneumocyte effector cell functions [17–19]. A549 cells were grown as a monolayer on 35-mm tissue culture dishes in the presence or absence of a variety of LPSs, serotypes 0111:B4 (Difco), 026:B6 (Sigma), and 055:B5 (Sigma), and 0127:B8 (Difco) for 72 h [23]. In order to do this, various concentrations of A549 cell supernatant fluids (1:27, 1:9, 1:3, 1:1, 1:0.3) were added into the cell culture wells. After 4–6 days in culture, the cells reached confluence and were then used for experiments.

The effect of various LPSs from *E. coli* serotypes and *P. aeruginosa* was assessed on other lung cell types: BEAS-2B cells and lung fibroblasts (HLFs; human lung, diploid, passage 27 (ATCC)), as previously reported [21]. The preparation routinely consisted of 30% monocytes and 70% lymphocytes, determined via morphology and α-naphthyl acetate esterase (Sigma) staining, with >98% viability, as assessed by trypsin blue and erythromycin exclusion. The cells were suspended in Gey’s balanced salt solution containing 2% bovine serum albumin at pH 7.2 to give a final concentration of 5.0×10^6 cells·mL⁻¹. These suspensions were used in the chemotaxis assay.

The chemotaxis assay was performed in 48-well microchemotaxis chambers (Neuroprobe Inc., Cabin John, MD, USA) as previously described [22]. Each sample was tested in duplicate. A polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA) with a pore size of 5 µm was placed over the bottom wells. The chamber was incubated in a humidified 5% CO₂ atmosphere for 90 min at 37°C. Cells that completely migrated through the filter were counted from five random high-power fields (HPFs, 60 x magnification) and the number of migrated cells was defined as the mean number of migrated cells per HPF. The chemotaxis assay was performed in 48-well microchemotaxis chambers (Neuroprobe Inc., Cabin John, MD, USA) as previously described [22]. Each sample was tested in duplicate. A polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA) with a pore size of 5 µm was placed over the bottom wells. The chamber was incubated in a humidified 5% CO₂ atmosphere for 90 min at 37°C. Cells that completely migrated through the filter were counted from five random high-power fields (HPFs, 60 x magnification) and the number of migrated cells was defined as the mean number of migrated cells per HPF.

### Exposure of A549 cells, BEAS-2B cells, and human foetal lung fibroblasts to lipopolysaccharides

Medium was removed from cells by washing twice with serum-free F-12, and cells were incubated with F-12 without foetal calf serum (FCS) in the presence or absence of a variety of *E. coli* LPSs, serotypes 0127:B8 (Difco, Detroit, MI, USA) and Sigma, St Louis, MO, USA), 0111:B4 (Sigma), 055:B5 (Sigma), and 026:B6 (Sigma), and *P. aeruginosa* LPS, serotype 10 (Sigma), at concentrations of 0, 0.01, 0.1, 1.0, and 100 µg·mL⁻¹ and cultured for 12, 24, 48, 72 and 96 h. In some experiments, A549 cells were stimulated with LPSs in the presence of 10% FCS. LPSs from Sigma were prepared by extraction into phenol by means of the method of Westphal et al. [2]. However, the content of lipid A was not determined. LPS from Difco was similarly prepared by phenol extraction, and the content of lipid A was 9.7%. LPS obtained from Sigma was tested for biological activity using LAL (table 1). No LPS caused injury (no deformity of cell shape, no detachment from tissue culture dish and >95% of cells viable by trypan blue exclusion) to A549, BEAS-2B and HLF cells after 72 h incubation at maximal concentrations. The culture supernatant fluids were harvested and frozen at -80°C until assayed. At least seven separate A549 cell supernatant fluids were harvested for each experimental condition.

### Measurement of monocyte chemotactic activity

Mononuclear cells were obtained for the chemotactic assay by Ficoll/Hypaque density centrifugation (Histopaque 1077, Sigma), as previously reported [21]. The preparation routinely consisted of 30% monocytes and 70% lymphocytes, determined via morphology and α-naphthyl acetate esterase (Sigma) staining, with >98% viability, as assessed by trypsin blue and erythromycin exclusion. The cells were suspended in Gey’s balanced salt solution containing 2% bovine serum albumin at pH 7.2 to give a final concentration of 5.0×10^6 cells·mL⁻¹. These suspensions were used in the chemotaxis assay.

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<table>
<thead>
<tr>
<th>LAL activity EU·mg⁻¹</th>
<th>Lipid A content %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> LPS</td>
<td>4×10⁶</td>
</tr>
<tr>
<td><em>E. coli</em> LPS</td>
<td></td>
</tr>
<tr>
<td>0127:B8 (Difco)</td>
<td>12.5×10⁶</td>
</tr>
<tr>
<td>0127:B8 (Sigma)</td>
<td>12.5×10⁶</td>
</tr>
<tr>
<td>0111:B4</td>
<td>3.0×10⁶</td>
</tr>
<tr>
<td>055:B5</td>
<td>1.0×10⁶</td>
</tr>
<tr>
<td>026:B6</td>
<td>2.0×10⁶</td>
</tr>
</tbody>
</table>

EU: endotoxin unit; ND: not determined.
1:1) were placed above the membrane with cells and below the membrane to establish a variety of concentration gradients across the membrane.

To ensure that monocytes, but not lymphocytes, were the primary cells that migrated, some of the membranes were stained with α-naphthyl acetate esterase, according to the manufacturer’s instructions.

**Partial characterization of monocyte chemotactic activity**

Partial characterization of MCA was performed using supernatant fluids harvested at 72 h incubation at a concentration of 100 μg·mL⁻¹. *E. coli* LPS 0127:B8 (Difco). Sensitivity to protease was tested using trypsin treatment (Sigma; final concentration 100 μg·mL⁻¹) for 30 min at 37°C followed by the addition of a 1.5 M excess of soybean trypsin inhibitor (Sigma) to terminate the proteolytic activity before the chemotaxis assay. The lipid solubility of the activity was evaluated by mixing the A549 cell culture supernatant fluid with ethyl acetate twice, decanting the lipid phase after each extraction, evaporating the ethyl acetate to dryness and resuspending the extracted material in F-12 before a chemotaxis assay. Heat sensitivity was determined by heating the culture supernatant fluid for 30 min at 98°C.

**Partial purification of the chemotactic activity by column chromatography**

In order to determine the approximate molecular weight of the released MCA, the supernatant fluids harvested at 72 h in response to 100 μg·mL⁻¹ *E. coli* LPS 0127:B8 (Difco) was examined by means of molecular sieve column chromatography using Sephadex G-100 (50 × 1.25 cm, Pharmacia, Piscataway, NJ, USA) at a flow rate of 6 mL·h⁻¹. The A549 culture supernatant fluid was eluted with phosphate-buffered saline, and every fraction after the void volume was evaluated for MCA in duplicate. The molecular weight markers were bovine serum albumin (66 kDa), cytochrome c (12.3 kDa) and quinacrine (450 Da).

**Effects of metabolic inhibitors on monocyte chemotactic activity release**

The effects of the nonspecific lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA; Sigma; 100 μM) and diethylcarbamazine (DEC; Sigma, 1 mM), and the 5-lipoxygenase inhibitor, AA-861 (Sigma; 100 μM) were evaluated. The effect of the protein synthesis inhibitor, cycloheximide (Sigma; 10 μg·mL⁻¹) was also assessed. At these concentrations, NDGA, DEC and AA-861 inhibited the release of leukotriene B₄ (LTB₄) in other cell cultures in response to *E. coli* LPS 0127:B8 (Difco) without serum, at the suggested concentrations to inhibit MCP-1, GM-CSF and TGF-β. The mixture of supernatant fluids and antibodies were incubated for 30 min in 37°C. These samples were then used for chemotactic assays. These antibodies did not influence the chemotactic response of monocytes to endotoxin-activated serum (data not shown). To exclude the nonspecific effects of immunoglobulin G (IgG), nonimmune IgG (Genzyme) was used as control. The nonimmune IgG did not affect monocyte chemotaxis in response to LPS-stimulated A549 cell supernatant fluids and endotoxin-activated serum (data not shown).

**Measurement of leukotriene B₄ and platelet activating factor in released monocyte chemotactic activity**

The LTB₄ receptor antagonist, ONO 4057, (Ono Pharmaceutical Co., Tokyo, Japan) and the platelet-activating factor (PAF) antagonist, TCV 309, (Takeda Pharmaceutical Co., Tokyo, Japan) at a concentration of 10⁻² M were used to evaluate the responsible MCA in the crude supernatant and the column chromatography-separated lowest molecular weight peak.

**Measurement of leukotriene B₄ and platelet activating factor in the supernatant fluid**

The measurement of LTB₄ was performed by means of radioimmunoassay, as previously described [20]. PAF concentration in the supernatant fluids was measured by means of a scintillation proximity assay system [20]. This system combined the use of a high-specific-activity triated PAF tracer with an antibody specific for PAF and a PAF standard, similar to the methods of measurement of LTB₄.

**Effects of polyclonal antibodies to monocyte chemotactant protein-1, granulocyte-macrophage colony-stimulating factor and transforming growth factor-β**

The neutralizing antibodies to monocyte chemotactant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor-β (TGF-β) were purchased from Genzyme (Cambridge, MA, USA). MCP-1, GM-CSF and TGF-β antibodies were added to the A549 cell supernatant fluids, which were cultured for 72 h at a concentration of 10 μg·mL⁻¹ *E. coli* LPS 0127:B8 (Difco) without serum, at the suggested concentrations to inhibit MCP-1, GM-CSF and TGF-β. The mixture of supernatant fluids and antibodies were incubated for 30 min in 37°C. These samples were then used for chemotactic assays. These antibodies did not influence the chemotactic response of monocytes to endotoxin-activated serum (data not shown). To exclude the nonspecific effects of immunoglobulin G (IgG), nonimmune IgG (Genzyme) was used as control. The nonimmune IgG did not affect monocyte chemotaxis in response to LPS-stimulated A549 cell supernatant fluids and endotoxin-activated serum (data not shown).

**Measurement of monocyte chemotactant protein-1 and granulocyte-macrophage colony-stimulating factor in supernatant fluids**

The concentrations of MCP-1 and GM-CSF in A549 cell supernatant fluids were measured by means of enzyme-linked immunosorbent assay, according to the manufacturer’s instructions. A549 cells were stimulated for 24 h at a concentration of 100 μg·mL⁻¹ *E. coli* LPSs 0127:B8
(Difco), 0127:B8 (Sigma), 0111:B4 (Sigma), 055:B5 and 026:B6, and *P. aeruginosa* LPS serotype 10 without serum. In other sets of experiments, A549 cells were stimulated for 72 h at a concentration of 10 μg·mL⁻¹ of various kinds of LPS in the presence and absence of 10% FCS. MCP-1 and GM-CSF kits were purchased from R&D Systems (Minneapolis, MN, USA) and Amersham (Amersham, UK), and the minimum concentrations detected for MCP-1 and GM-CSF were 10.0 pg·mL⁻¹ and 2.0 pg·mL⁻¹, respectively.

**Statistics**

In experiments in which multiple measurements were made, differences between groups were tested for significance using one-way analysis of variance, with Duncan’s multiple range test applied to the data at specific time-points and concentrations. In experiments in which single measurements were made, the differences between groups were tested for significance using Student’s paired t-test. In all cases, a p-value <0.05 was considered significant. Data in figures and tables are expressed as means±SEM.

**Results**

**Release of monocyte chemotactic activity from A549 cell, BEAS-2B cell and human foetal lung fibroblast monolayers**

LPSs stimulated the release of MCA from A549 cells in a dose- and time-dependent manner. However, there was difference among *E. coli* serotypes and species (*P. aeruginosa* >0127:B8 (Difco) >026:B6; p<0.01 between each LPS; fig. 1a and b, table 2). In contrast, LPSs derived from *E. coli* 0127:B8 (Sigma) and 0111:B4 did not stimulate A549 cells to release MCA. HLFs and BEAS-2B cells responded similarly to A549 cells to various kinds of LPS (table 3). The release of MCA from HLFs and BEAS-2B cells was stimulated for 72 h at various LPS concentrations (n=8). Compared with various LPSs, the release of MCA from A549 cells to various kinds of LPSs progressively increased the release of MCA up to 100 μg·mL⁻¹. The release of MCA began 12 h after exposure to LPS, and the released activity was cumulative (fig. 1b), even after 72 h. The chemotactic activities in response to FMLP and activated serum were 57.4±6.7 and 64.6±6.3 monocytes·HPF⁻¹, respectively.

**Table 2.** Release of monocyte chemotactic activity (MCA), monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) from A549 cells in response to a variety of lipopolysaccharides (LPSs)

<table>
<thead>
<tr>
<th>LPS Type</th>
<th>MCA cells·HPF⁻¹</th>
<th>MCP-1 pg·mL⁻¹</th>
<th>GM-CSF pg·mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>65.4±4.2*</td>
<td>17608±423*</td>
<td>5.8±2.7*</td>
</tr>
<tr>
<td><em>E. coli</em> LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0127:B8 (Difco)</td>
<td>43.2±3.8*</td>
<td>2678±498*</td>
<td>3.8±0.4*</td>
</tr>
<tr>
<td>0127:B8 (Sigma)</td>
<td>25.2±3.1</td>
<td>1012±213</td>
<td>2.1±0.5*</td>
</tr>
<tr>
<td>0111:B4</td>
<td>25.8±3.5</td>
<td>912±59</td>
<td>2.4±0.6*</td>
</tr>
<tr>
<td>055:B5</td>
<td>35.5±2.4*</td>
<td>2815±421*</td>
<td>2.2±0.5*</td>
</tr>
<tr>
<td>026:B6</td>
<td>28.9±3.2*</td>
<td>2325±210*</td>
<td>3.2±2.6*</td>
</tr>
<tr>
<td>F-12</td>
<td>22.6±2.1</td>
<td>891±51</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM (n=9). A549 cells were incubated with various LPSs at a concentration of 100 μg·mL⁻¹ without foetal calf serum for 72 h to determine MCA concentration and for 24 h to determine MCP-1 and GM-CSF concentration. ND: not determined. *: p<0.01 versus F-12 (negative control).

**Confirmation that the migrated cells were monocytes**

Checkerboard analysis revealed that A549 cell supernatant fluids stimulated by *E. coli* LPS 0127:B8 (Difco) induced monocyte migration in the presence of a concentration gradient across the membrane, but induced weak migration without a gradient (table 4), suggesting that the migration was a chemotactic rather than a chemokinetic activity.

**Confirmation that the migrated cells were monocytes**

Confirmation that the migrated cells were monocytes was provided by the following lines of evidence: 1) >90% of the migrated cells appeared to be monocytes on light microscopy; 2) >90% of migrated cells were esterase-positive; and 3) lymphocytes purified by allowing monocytes to attach to plastic and tested in the chemotaxis assay yielded 0–20% of the chemotactic activity of the monocyte preparation.

![Fig. 1.](image-url) Release of monocyte chemotactic activity (MCA) in response to *Pseudomonas aeruginosa* lipopolysaccharide (LPS) serotype 10 (●), *Escherichia coli* LPS 0127:B8 (Difco, ▲) and *E. coli* LPS 026:B6 (▲) from A549 cell monolayers after: a) incubation for 72 h at various LPS concentrations (n=8), and b) incubation at 100 μg·mL⁻¹ LPS for various times. Data are expressed as means±SEM.
Inhibitory effect of polymyxin B on the release of monocyte chemotactic activity from A549 cells, human foetal lung fibroblasts (HLFs) and BEAS-2B cells

Polymyxin B almost completely inhibited the release of MCA from A549 cells, HLFs and BEAS-2B cells in response to 100 μg·mL⁻¹ LPSs derived from *P. aeruginosa* and *E. Coli* 0127:B8 (Difco), 055:B5 and 026:B6 (table 3).

Polymyxin B per se did not affect the monocyte chemotactic response to activated serum and FMLP.

Partial characterization of the released monocyte chemotactic activity

The MCA released from A549 cells was heterogeneous in character. It was sensitive to heat, extractable into ethyl acetate and partially digested by trypsin (fig. 2). Incubation of A549 cells with cycloheximide inhibited the release of MCA (fig. 3). The nonspecific lipoxygenase inhibitors, NDGA and DEC, and the 5-lipoxygenase inhibitor, AA-861, attenuated the release of MCA (*p* < 0.01; fig. 3). NDGA, DEC and AA-861 did not have any effects on FMLP and activated serum-induced monocyte chemotaxis (data not shown).

Partial purification of monocyte chemotactic activity

Sephadex-100 MCA purification revealed that MCA was heterogeneous in size, with estimated molecular masses of 70, 26, 16, 8 and 0.4 kDa (fig. 4). MCA of 26 and 17 kDa were predominant.

Effects of leukotriene B₄ and platelet-activating factor receptor antagonist on monocyte chemotactic activity

MCA in the supernatant fluids and the lowest molecular mass activity separated by molecular sieve column...
completely inhibited the monocyte migration in response to FMLP and activated serum-induced monocyte chemotaxis (data not shown).

From A549 cells in response to LPSs derived from *P. aeruginosa* B5, respectively (data not shown).

The effect of the PAF receptor antagonist, TCV 309, on MCA was not significant (table 5). The LTB4 receptor antagonist inhibited MCA in the supernatant fluids released from A549 cells in response to LPSs derived from *P. aeruginosa*, *E. coli* 026:B6, 0127:B8 (Sigma) and 055:B5, respectively (data not shown).

Each receptor antagonist at a concentration of 10^-5 M completely inhibited the monocyte migration in response to LTB4 and PAF at a concentration of 10^-8 M, but did not affect FMLP and activated serum-induced monocyte chemotaxis (data not shown).

Concentrations of leukotriene B4 and platelet-activating factor receptor antagonists (TCV 309) in the supernatant fluids

The concentrations of LTB4 in the supernatant fluids in response to *E. coli* LPS serotype 0127:B8 (Difco) at a concentration of 100 μg·mL^-1 for 72 h and control were 68.9±15.4 versus 55.4±13.4 ng·mL^-1 (n=8, p>0.05). However, PAF was not detected in the supernatant fluids in response to LPS (<40 pg·mL^-1).

Effects of blocking antibodies to monocyte chemoattractant protein-1 and granulocyte-macrophage colony-stimulating factor on monocyte chemotactic activity in the supernatant fluids

Anti-MCP-1 significantly blocked the chemotactic response to monocytes. The inhibition of total MCA by anti-MCP-1 antibody was 60% (fig. 5). Anti-MCP-1 inhibited the 16 kDa chemotactic activity separated by molecular sieve chromatography by 70%. Anti-GM-CSF antibody inhibited the MCA in the supernatant fluids by 20% (fig. 5) and the 26 kDa chemotactic activity by 80%. In contrast, anti-TGF-β did not attenuate MCA. These antibodies inhibited MCA released from A549 cells in response to LPSs derived from *P. aeruginosa* 0127:B8 (Sigma), 055:B5, and 026:B6, respectively (data not shown). Anti-MCP-1, anti-GM-CSF, and the LTB4 receptor antagonist together inhibited total MCA by up to 80% (fig. 5).

Concentrations of monocyte chemoattractant protein-1 and granulocyte-macrophage colony-stimulating factor in the supernatant fluids

The measurement of MCP-1 and GM-CSF revealed that incubation with LPSs at a concentration of 100 μg·mL^-1 for 24 h significantly stimulated the release of MCP-1 and GM-CSF (*P. aeruginosa* 0127:B8 (Difco)=055:B5= 026:B6 for MCP-1, *P. aeruginosa* 0127:B8 (Difco)=026:B6 for GM-CSF; table 2). LPS 0127:B8 (Sigma) and 0111:B4 did not stimulate the release of MCP-1 (table 2). LPSs derived from 055:B5, 0127:B8 (Sigma) and 0111:B4 stimulated slight release of GM-CSF. As previously noted, this concentration of LPS was not cytotoxic to A549 cells. LPSs stimulation at a concentration of 10 μg·mL^-1 for 72 h without serum caused a small increase in the release.
**Discussion**

The cytotoxicity of LPS derived from *P. aeruginosa* is less potent than that from *E. coli* 0127:B8. However, *P. aeruginosa* infection induces prominent sustained inflammation in the lung, including in cystic fibrosis, diffuse panbronchiolitis and chronic inflammatory lung diseases. LPS from *P. aeruginosa* stimulated airway epithelial cells and fibroblasts to release MCA, i.e. MCP-1 and GM-CSF more potently than did *E. coli* LPSs. The high potential for *P. aeruginosa* LPS to stimulate lung cells may, at least partly, explain the prominent sustained lung inflammation observed at sites of *P. aeruginosa* infection.

It is reported that A549 cells release MCP-1 in response to tumour necrosis factor (TNF) and interleukin (IL)-1 [19]. However, *E. coli* LPS serotype 0111:B4 did not stimulate the release of MCP-1 from A549 cells in the previous study [19]. The stimulatory potential of *E. coli* LPS 0127:B8 (Sigma) and 0111:B4 to release MCA was not significant in the present study. LPSs from different *E. coli* serotypes and *P. aeruginosa* stimulated the release of MCA, MCP-1 and GM-CSF from A549 cells. The presence of serum augmented the release of MCP-1 and GM-CSF. But, differences among the LPSs from *E. coli* serotypes and *P. aeruginosa* were observed. Thus, the response of lung cells to LPSs may be differently regulated depending on the *E. coli* serotypes or species involved.

The potential for LPS from *P. aeruginosa* to stimulate lung cells was most prominent. The differing stimulatory potential among LPSs from *E. coli* serotypes to release MCA was evident. The release of MCP-1 or GM-CSF was regulated by LPSs, dependent on serotype and species. Because MCA consisted predominantly of MCP-1 and GM-CSF, the relations among MCA, MCP-1 and GM-CSF correlated closely with each other. These data suggest that the differing stimulatory potential of MCA among LPSs may be applied to the release of MCP-1 and GM-CSF.

*P. aeruginosa* LPS had a higher potential than the *E. coli* LPSs examined. However, the LAL activity of *P. aeruginosa* LPS was less than that of *E. coli* LPS 0127:B8. Since polymyxin B blocked the effects of the LPSs, the possibility of contaminating molecules in LPSs stimulating lung epithelial cells and fibroblasts was low. The cytotoxicity of *P. aeruginosa* LPS is reported to be less than that of *E. coli* LPS by virtue of its specific lipid A structure [8, 10]. Thus, the cytotoxic potential of LPSs, i.e. LAL activity may not correlate with the stimulatory potential of LPS to release MCA from lung epithelial cells.

The release of MCA in response to *P. aeruginosa* LPS was increased four–five-fold compared with the constitutive release of MCA. The releasing potential of MCA from A549 cells in response to *P. aeruginosa* was more than that from 10^5 alveolar macrophages per culture dish in response to IL-1, TNF and LPS (data not shown). Moreover, the release of MCA, MCP-1 and GM-CSF by *P. aeruginosa* LPS from A549 cells were almost the same as those from A549 cells stimulated with 500 pg·mL^{-1} of IL-1β and 1,000 U·mL^{-1} TNF-α (data not shown). Thus, the results of the present study suggest that *P. aeruginosa* LPS may contribute to the recruitment of inflammatory cells into the lung by stimulating lung epithelial cells and fibroblasts.

The concentration of LPSs required to stimulate A549 cells, BEAS-2B cells and HLFs was greater than that required for monocyte/macrophage stimulation. Although the concentration of LPSs at sites of bacterial infection or colonization is uncertain, the LPS concentration in bronchoalveolar lavage fluids (BALFs) from patients with adult respiratory distress syndrome was 1–1,585 pg·mL^{-1} [26]. Since the BALF was diluted 50–100 times, the local...
concentration of LPS at the sites of bacterial infection and colonization would be far higher than that generally found in epithelial lining fluid. Thus, the high concentration of LPS at the sites of bacterial infection and colonization would be far higher than that generally accessible at the site of bacterial infection and colonization.

In conclusion, lipopolysaccharide derived from \textit{Pseudomonas aeruginosa} stimulated lung epithelial cells and fibroblasts to release monocyte chemotactic activity more potently than lipopolysaccharide derived from \textit{Escherichia coli}. Although the release of monocyte chemoattractant protein-1 and granulocyte-macrophage colony-stimulating factor was augmented by the addition of serum, differing stimulatory potential among lipopolysaccharides was observed. These data suggest that \textit{Pseudomonas aeruginosa} lipopolysaccharide may induce the sustained prominent lung inflammation, observed at sites of \textit{Pseudomonas aeruginosa} infection.

References


