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

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The potential of various lipopolysaccharides to release monocyte chemotactic activity from lung epithelial cells and fibroblasts. S. Koyama, E. Sato, H. Nomura, K. Kubo, M. Miura, T. Yamashita, S. Nagai, T. Izumi. ©ERS Journals Ltd 1999.
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:B5 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-GSF. 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.
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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 β1–6-linked D-glucohexosamine 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-acyloxyacyl groups in a defined structural arrangement (as in *Escherichia coli*) is a prerequisite [4].

However, the lipid A structure of some nonenterobacterial LPSs have 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.

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 assess type II pneumocyte effector cell functions [17–19]. A549 cells were grown as a monolayer on 35-mm tissue culture dishes as has been previously reported [20]. After cells reached confluence, they were used for the experiments.

The effect of various LPSs from *E. coli* serotypes and *P. aeruginosa* were assessed on other lung cell types: BEAS-2B cells (a kind gift from C. Harris, National Institute of Health, Bethesda, MD, USA) and human foetal lung fibroblasts (HLFs; human lung, diploid, passage 27 (ATTC)), which were established as a cell line and commercially available. BEAS-2B cells and HLFs were cultured in Ham's F-12 medium at 37°C in 5% CO_2 on 35-mm-diameter tissue culture dishes. After 4–6 days in culture, the cells reached confluence and were then used for experiments.

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, 0.1, 1.0, 10 and 100 $\mu g \cdot mL^{-1}$ 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 trypan 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% CO_2 atmosphere for 90 min at 37°C. Cells that completely migrated through the filter were counted from five random high-power fields (HPFs, ×1,000) from each duplicate well. Chemotactic response was defined as the mean number of migrated cells·HPF^{-1}. F-12 without FCS was incubated identically with A549 cells, and the supernatant fluids harvested were used to determine background monocyte migration. *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma, 10^{-8} M in F-12) and normal human serum, which was complement-activated by incubation with *E. coli*, LPS 0127:B8 (Difco) and diluted 10-fold with F-12, were used as positive controls.

To determine whether the migration was due to movement along a concentration gradient (chemotaxis) or stimulation to randomly migrate (chemokinesis), a checkerboard analysis was performed using A549 cell supernatant fluid stimulated by 100 $\mu g \cdot mL^{-1}$ *E. coli* LPS serotype 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,

Table 1. – *Limulus amoebocyte* lysate (LAL) activity and lipid A content of lipopolysaccharide (LPS)

	LAL activity EU·mg^{-1}	Lipid A content %
P. aeruginosa LPS	4.0×10^6	ND
E. coli LPS		
0127:B8 (Difco)	12.5×10^6	9.7
0127:B8 (Sigma)	12.5×10^6	ND
0111:B4	3.0×10^6	ND
055:B5	1.0×10^6	ND
026:B6	2.0×10^6	ND

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 (Takeda Pharmaceutical Co., Tokyo, Japan; 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 B$_4$ (LTB$_4$) in other cell cultures in response to *E. coli* LPS 0127:B8 (Difco) [24, 25] and did not cause cytotoxicity to A549 cells after a 72-h incubation.

Effects of polymyxin B on the release of monocyte chemotactic activity from A549 cells, BEAS-2B cells and human foetal lung fibroblasts in response to lipopolysaccharides

The remarkably high concentrations of LPSs (10–100 μg·mL^{-1}) required to cause substantial effects were 100–1,000 times greater than that required for monocyte/macrophage effects. While this might reflect biological differences in receptors and signalling, it raised the possibility that contaminating molecules within the LPS preparation were responsible for the observed effects. To exclude the possibility of contaminant molecules causing the observed effects, polymyxin B (Sigma; 10 μg·mL^{-1}) was used to abrogate the effects of LPSs on the release of MCA from A549 cells, BEAS-2B cells and HLFs.

Role of leukotriene B$_4$ and platelet-activating factor in released monocyte chemotactic activity

The LTB$_4$ 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^{-5} M were used to evaluate the responsible MCA in the crude supernatant and the column chromatography-separated lowest molecular weight peak.

Measurement of leukotriene B$_4$ and platelet activating factor in the supernatant fluid

The measurement of LTB$_4$ 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 tritiated PAF tracer with an antibody specific for PAF and a PAF standard, similar to the methods of measurement of LTB$_4$.

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

The neutralizing antibodies to monocyte chemoattractant 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 chemoattractant 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), 0111B4 (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 $\mu g \cdot mL^{-1}$ 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 $pg \cdot mL^{-1}$ and 2.0 $pg \cdot mL^{-1}$, 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 in the order *P. aeruginosa* >0127:B8 (Difco) >026:B6, (p<0.01 between each LPS, data not shown). LPS derived from *E. coli* 0111:B4 did not stimulate HLFs and BEAS-2B cells. The lowest dose of LPSs

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)

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

Data are presented as mean±SEM (n=9). A549 cells were incubated with various LPSs at a concentration of 100 $\mu g \cdot mL^{-1}$ 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).

to stimulate A549 cells was 0.1 $\mu g \cdot mL^{-1}$. Increasing concentrations of LPS progressively increased the release of MCA up to 100 $\mu g \cdot mL^{-1}$. 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.

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 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.
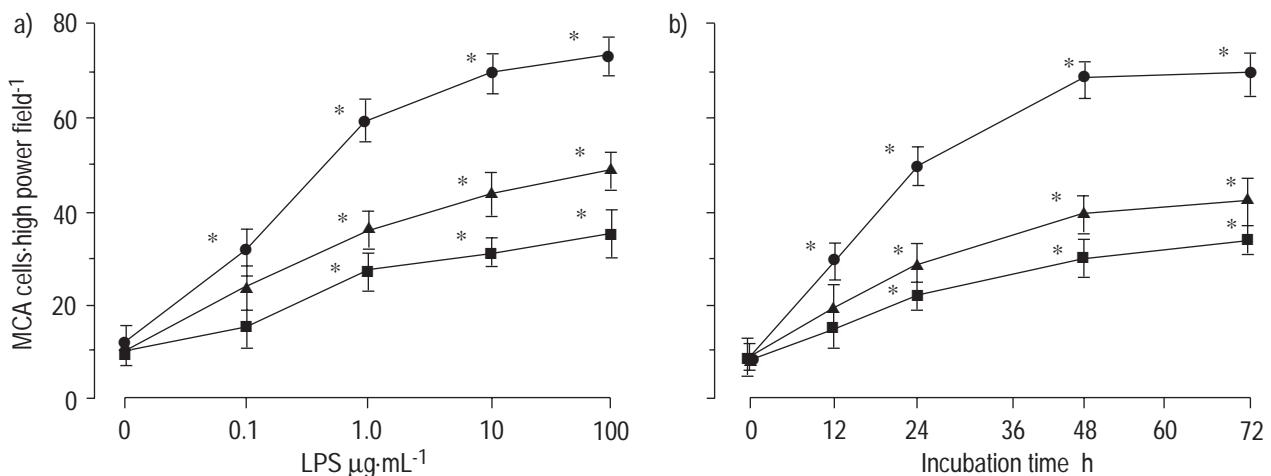

Fig. 1. – Release of monocyte chemotactic activity (MCA) in response to *Pseudomonas aeruginosa* lipopolysaccharide (LPS) serotype 10 (●), *Escherichia coli* LPS 0127:B8 (Difco, ▲) and *E.coli* LPS 026:B6 (■) from A549 cell monolayers after: a) incubation for 72 h at various LPS concentrations (n=8); and b) incubation at 100 $\mu g \cdot mL^{-1}$ LPS for various times. Data are expressed as mean±SEM.

Table 3. – Inhibition of monocyte chemotactic activity by polymyxin B from A549 cells, BEAS-2B cells and human foetal lung fibroblasts (HLFs) in response to a variety of lipopolysaccharides (LPSs)

	PMB-	PMB+
A549 cells		
P. aeruginosa LPS	65.4±4.2*	14.3±4.2
E. coli LPS		
0127:B8 (Difco)	43.2±3.8*	12.4±2.1
055:B5	35.5±2.4	10.3±2.5
026:B6	28.9±3.2	12.8±3.1
BEAS-2B cells		
P. aeruginosa LPS	5.7±2.8	10.2±3.2
E. coli LPS		
0127:B8 (Difco)	34.7±2.4	11.3±3.1
055:B5	24.5±2.1	12.4±3.2
026:B6	25.9±2.4	9.8±2.9
HLFs		
P. aeruginosa LPS	48.8±2.3	9.3±2.2
E. coli LPS		
0127:B8 (Difco)	39.6±3.5	10.5±3.0
055:B5	22.8±2.5	10.9±3.1
026:B6	24.4±3.1	11.2±2.5

Data are presented as mean±SEM (n=9). A549 cells, BEAS-2B cells and fibroblasts were incubated with various LPSs at a concentration of 100 μg·mL^{-1} for 72 h in the presence (PMB+) or absence of polymyxin B (PMB-) at a concentration of 10 μg·mL^{-1}.

Inhibitory effect of polymyxin B on the release of monocyte chemotactic activity from A549 cells, human foetal lung fibroblasts, 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

Table 4. – Checkerboard analysis of released monocyte chemoattractant activity in response to *Escherichia coli* lipopolysaccharide (LPS) serotype 0127:B8

	Upper well				
	Supernatant fluid dilution				
Lower well	F-12	1:27	1:9	1:9	1:9
F-12	6.5±1.3	12.1±2.1	13.6±2.8	16.5±2.1	20.1±2.2
Supernatant fluid dilution					
1:27	8.5±2.1	12.4±1.9	15.2±2.8	17.6±2.5	21.8±2.3
1:9	13.6±2.2	18.2±2.9	23.5±2.7	23.7±3.6	24.5±3.2
1:3	20.3±2.2	19.4±3.5	20.4±3.5	21.3±3.6	24.3±3.1
1:1	386±2.6	34.6±3.5	29.7±2.7	24.5±2.7	23.5±2.5

Values are mean±SD cells·high power field^{-1}. Checkerboard analysis of A549 cell culture supernatant fluid harvested after 72 h in response to *E. coli* LPS 0127:B8 (Difco) at a concentration of 100 μg·mL^{-1}. F-12 was used as a negative control (without foetal calf serum).
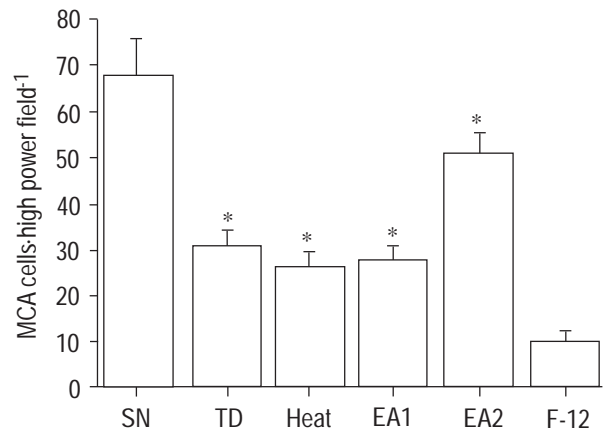
Fig. 2. – Partial characterization of the monocyte chemotactic activity (MCA) released in response to *Escherichia coli* lipopolysaccharide serotype 0127:B8 (Difco) in A549 cell supernatant fluids harvested after 72 h incubation. Values are expressed as mean±SEM (n=6). F-12 medium served as negative control. SN: supernatant; TD: trypsin digest; EA1: ethyl acetate extract; EA2: non-ethyl acetate extracted.

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 B$_4$ 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
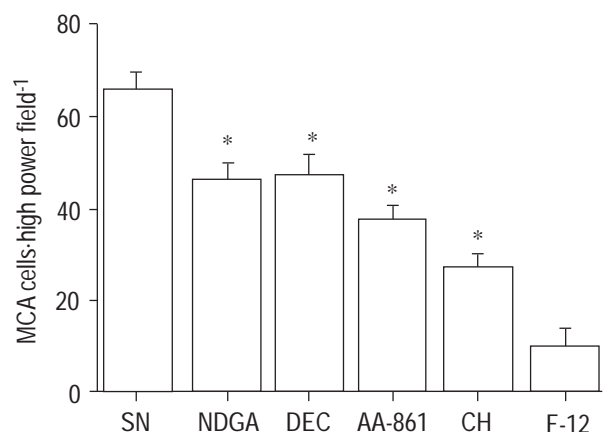
Fig. 3. – Effect of nordihydroguaiaretic acid (NDGA), diethylcarbamazine (DEC), AA-861 and cycloheximide (CH) on the release of monocyte chemotactic activity (MCA) in response to *Escherichia coli* lipopolysaccharide serotype 0127:B8 (Difco). F-12 medium served as negative control. Values are expressed as mean±SEM (n=5). SN: cell supernatant. *: p<0.01 *versus* stimulus alone.
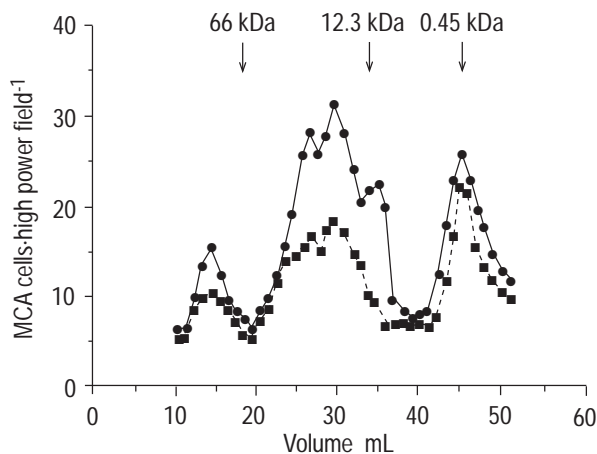
Fig. 4. – Molecular sieve column chromatographic analysis of monocyte chemotactic activity (MCA) released from A549 cell monolayers in the unstimulated state (- -■- -) and in response to incubation with 100 µg·mL⁻¹ *Escherichia coli* lipopolysaccharide serotype 0127:B8 for 72 h (—●—). Values are representative data from four different supernatants fluids. Molecular weight markers were: bovine serum albumin 66 kDa, cytochrome c 12.3 kDa, and quinacrine 0.45 kDa. Fraction volume collected is 1.0 mL.

chromatography were inhibited by the addition of the LTB$_4$ receptor antagonist, ONO 4057, by approximately 25 and 60%, respectively (p<0.01; fig. 5, table 5). The effect of the PAF receptor antagonist, TCV 309, on MCA was not significant (table 5). The LTB$_4$ 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 LTB$_4$ and PAF at a concentration of 10^{-7} M, but did not affect FMLP and activated serum-induced monocyte chemotaxis (data not shown).
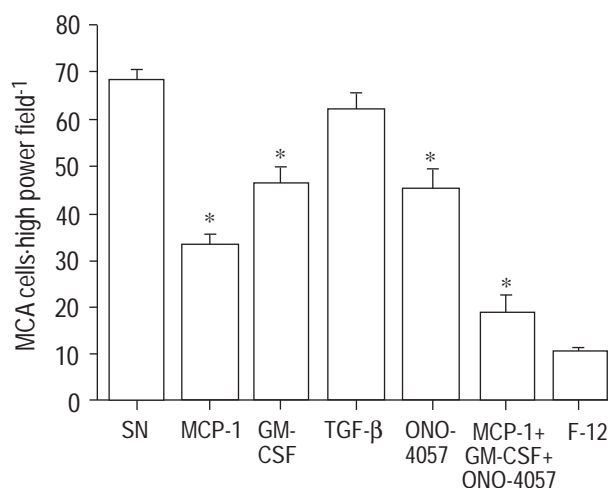

Fig. 5. – Effects of antibodies directed against monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor-β (TGF-β) and the leukotriene B$_4$ receptor antagonist, ONO 4057, on monocyte chemotactic activity (MCA) in A549 cell supernatant fluid stimulated by *Escherichia coli* lipopolysaccharide serotype 0127:B8 for 72 h. F-12 medium served as negative control. Values are expressed as mean±SEM (n=6). *: p<0.01 *versus* untreated cell supernatant (SN).

Table 5. – Effects of leukotriene B$_4$ (ONO 4057) and platelet-activating factor receptor antagonists (TCV 309) on the lowest molecular mass column chromatography-separated monocyte chemotactic activity (MCA)

	MCA
MCA fraction	18.7±3.5
+ ONO 4057	12.5±2.2*
+ TCV 309	17.9±2.1
PBS	7.3±1.2

Values are mean±SEM cells·high power field⁻¹ (n=4). PBS: phosphate buffered saline served as negative control. *: p<0.01 *versus* MCA fraction.

Concentrations of leukotriene B$_4$ and platelet-activating factor in the supernatant fluids

The concentrations of LTB$_4$ 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 column 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 LTB$_4$ 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 0111B4 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
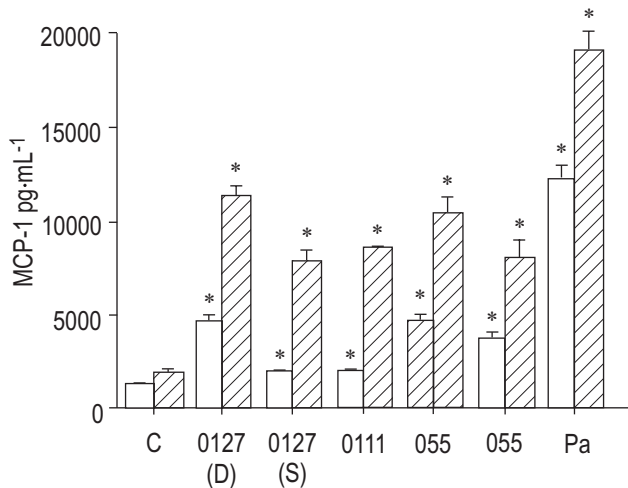
Fig. 6. – Release of monocyte chemoattractant protein-1 (MCP-1) from A549 cell monolayers in response to incubation with LPSs derived from *Escherichia coli* and *Pseudomonas aeruginosa* (Pa) at a concentration of 10 $\mu g \cdot mL^{-1}$ for 72 h in the absence (□) and presence (▨) of serum. Data are presented as mean±SEM. (n=9). *E.coli* serotypes: 0127(D): 0127:B8 (Difco); 0127(S): 0127:B8 (Sigma); 0111: 0111:B4; 055: 055:B5; 026: 026:B6. *: p<0.01 *versus* control (C).

of MCP-1 and GM-CSF, dependent on the LPS used (*P. aeruginosa* >0127:B8 (Difco)=055:B5=026:B6 >0127: B8: 0111 for MCP-1 (fig. 6), *P. aeruginosa*=0127:B8 (Difco) >055:B5=026:B6=0127:B8 (Sigma)=0111:B4 for GM-CSF (fig. 7)). Although the release of MCP1 and GM-CSF was remarkably augmented by the addition of 10% FCS (figs. 6 and 7), the dependency on serotype and species was observed.

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
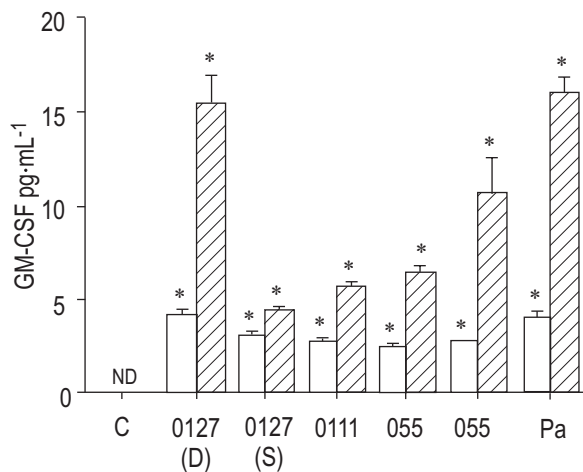

Fig. 7. – Release of granulocyte-macrophage colony-stimulating factor (GM-CSF) from A549 cell monolayers in response to incubation with LPSs derived from *Escherichia coli* and *Pseudomonas aeruginosa* (Pa) at a concentration of 10 $\mu g \cdot mL^{-1}$ for 72 h in the absence (□) and presence (▨) of serum. Data are presented as mean±SEM. (n=9). *E.coli* serotypes: 0127(D): 0127:B8 (Difco); 0127(S): 0127:B8 (Sigma); 0111: 0111:B4; 055: 055:B5; 026: 026:B6. *: p<0.01 *versus* control (C).

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 also 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 were 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 10^6 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 LPSs may be accessible at the site of bacterial infection and colonization.

In conclusion, lipopolysaccharide derived from *Pseudomonas aeruginosa* stimulated lung epithelial cells and fibroblasts to release monocyte chemotactic activity more potently than lipopolysaccharide derived from *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 *Pseudomonas aeruginosa* lipopolysaccharide may induce the sustained prominent lung inflammation, observed at sites of *Pseudomonas aeruginosa* infection.

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