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Monocyte chemotactic factors released from type II pneumocyte-like cells in response to TNF-α and IL-1α

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ABSTRACT: It has been reported that tumour necrosis factor (TNF)-α and interleukin (IL)-1 induce the release of monocyte chemotactic factors (MCF), including chemokines, from A549 cells, an alveolar type II cell line. However, the relative contribution of these chemokines to MCF is still uncertain.

In the present study, the relative contribution of various chemokines released from A549 cells acting as MCF upon stimulation by TNF-α and IL-1α, was evaluated.

TNF-α and IL-1α induced the release of MCF in a dose- and time-dependent manner ($p<0.001$). The release of MCF was inhibited by cycloheximide and lipoxygenase inhibitors. Molecular sieve column chromatography revealed multiple peaks of MCF (near 60 kDa, 25–22 kDa, 15–13 kDa, 8 kDa, and 400 Da). Leukotriene B_4 (LTB_4) receptor-antagonists inhibited MCF by 50% after 24 h and 30% after 72 h. Monocyte chemoattractant protein-1 (MCP-1), transforming growth factor (TGF)-β, "regulated on activation, normal T-cells, expressed and secreted" (RANTES), and granulocyte-macrophage colony- stimulating factor (GM-CSF) were released significantly in response to IL-1α and TNF-α, and antibodies to MCP-1, GM-CSF, and RANTES inhibited MCF activity by 40, 5 and 20% after 24 h, and by 50, 20, and 10% after 72 h, respectively. Each antibody or LTB_4 receptor-antagonist inhibited the corresponding column chromatography-separated molecular weight peak of MCF.

These data suggest that A549 cells release monocyte chemoattractant protein-1 as the predominant monocyte chemotactic factor rather than granulocyte-macrophage colony-stimulating factor, RANTES, and transforming growth factor-β, and that leukotriene B_4 is constitutively released as a monocyte chemotactic factor.
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The alveolar macrophage, an important phagocyte of the pulmonary airspace and the interstitium, is derived predominantly from differentiated peripheral blood monocytes and to a limited extent from local macrophage replication [1–3]. The recruitment of peripheral blood monocytes to the lung is essential for normal lung immune function but it is also involved in the generation and evolution of an inflammatory response to pulmonary injury. During acute and chronic inflammation, the process of monocyte elicitation is accelerated resulting in a transient or more prolonged increase in alveolar macrophages [4, 5]. Although elicited macrophages serve a vital role in the host defence against a number of organisms, the presence of increased numbers of activated macrophages can lead to excessive tissue injury *via* excessive elaboration of inflammatory cytokines, eicosanoids, proteolytic enzymes, and oxygen radicals [6–8].

Alveolar type II (ATII) epithelial cells have been shown to play a significant role in the regulation of the alveolar space. ATII cells synthesize and secrete surfactant, control the volume and composition of the epithelial lining fluid, and proliferate and differentiate into type I alveolar epithelial cells after lung injury in order to maintain the integrity of alveolar wall [9]. Moreover, ATII cells also play a role in modulating immunological activity in the alveolar space. In this setting, A549 cells, an ATII cell line, secreted monocyte chemoattractant activity constitutively [10] and released interleukin (IL)-8 [11, 12], IL-6 [13], interferon [9], and monocyte chemoattractant protein (MCP)-1 [14] in response to tumour necrosis factor (TNF)-α and IL-1α, suggesting participation in the intra-alveolar cytokine network. Transforming growth factor (TGF)-β [15], platelet-derived growth factor (PDGF) [16, 17], granulocyte-macrophage colony-stimulating factor (GM-CSF) [18–20] and "regulated on activation, normal T-cell expressed and secreted" (RANTES) [21] were detected in A549 cells *in vitro* or in the lung in patients with idiopathic pulmonary fibrosis. However, the relative contribution of these cytokines in their action as monocyte chemotactic factors (MCF) is uncertain.

In the present investigation, the relative potential of cytokines released from A549 cells to act as MCF was evaluated.

Materials and methods

Culture and identification of type II alveolar epithelial cells

Because of the difficulty in obtaining primary human type II epithelial cells of sufficient purity, A549 cells

(American Tissue Culture Collection, Rockville, MD, USA), an ATII cell line derived from an individual with alveolar carcinoma [22], was used. These cells retained many of the characteristics of normal type II cells such as surfactant protein secretion, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance and they have been extensively used to assess type II pneumocyte effector cell function [12–14]. A549 cells were grown as monolayers on 100-mm diameter tissue culture dishes. They were incubated in 100% humidity and 5% CO_2 at 37°C with F-12 medium supplemented with penicillin (50 U·mL^{-1}, GIBCO, Grand Island, NY, USA), streptomycin (50 μg·mL^{-1}, GIBCO), fungizone (2 μg·mL^{-1}, GIBCO) and 10% heat-inactivated foetal calf serum (FCS; GIBCO). The cells from monolayers were harvested with trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA; 0.1%) in phosphate-buffered saline (PBS), centrifuged at low speed (250 × *g*, 5 min), and resuspended in fresh medium at the concentration of 10^6 cells·mL^{-1} in 35-mm diameter tissue culture dishes. The cells were grown to confluence on the dish during 5–7 days. After reaching confluence, the cells were used for the experiment.

Exposure of A549 cells to IL-1α and TNF-α

Medium was removed from cells by washing twice with serum-free F-12, and cells were incubated with F-12 without FCS in the presence or absence of human recombinant IL-1α (0.5, 5.0, 50, 500 pg·mL^{-1}, Genzyme, Cambridge, MA, USA) and human recombinant TNF-α (1.0, 10, 100, and 1,000 U·mL^{-1}, Genzyme) and cultured for 12, 24, 48, 72, and 96 h. IL-1α and TNF-α were tested for endotoxin contamination. These cytokines did not cause A549 cell injury after 72 h incubation at the maximal doses (no deformity of cell shape, no detachment from tissue culture dish, and >95% of cells were viable by trypan blue exclusion). The culture supernatants were harvested and frozen at -80°C until assayed. At least seven separate A549 cell supernatants were harvested from cultures for each experimental condition.

Monocyte chemotactic assay

Human mononuclear cells were obtained for the chemotactic assay by Ficoll-Hypaque density centrifugation (Histopaque 1077, Sigma, St. Louis, MO, USA) to separate the red blood cells and neutrophils from mononuclear cells. The mononuclear cells were harvested at the interface, and the red blood cells were removed by suspending in the lysing buffer. The suspension was then centrifuged at 400 × *g* for 10 min and washed three times in Hanks' balanced salt solution (HBSS; GIBCO). The preparation routinely consisted of 30% monocytes and 70% lymphocytes as determined by morphology and α-naphthyl acetate esterase staining (Sigma) with >98% viability as assessed by trypan blue and erythrosin exclusion. The cells were suspended in Gey's balanced salt solution containing 2% bovine serum albumin (BSA; Sigma) at pH 7.2 to give a final concentration of 5.0×10^6 cells·mL^{-1}. This suspension was used in the chemotaxis assay.

The chemotaxis assay was performed in 48-well microchemotaxis chambers (Neuroprobe Inc., Cabin John, MD, USA) as has been described [23]. Each sample was tested in duplicate. After the bottom wells were filled with 25 μL of samples to test chemotactic activity, a polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA) with a pore size of 5 μm was placed over the bottom wells. Then 50 μL of mononuclear cell suspension was applied to the top wells. The chamber was incubated in humidified air (with 5% CO_2) at 37°C for 90 min. The chamber was disassembled after the incubation, and the filter was fixed, stained with Diff-Quik (American Scientific Products, McGraw Park, IL, USA) and mounted on a glass slide. Cells that completely migrated through the filter were counted in five random high power fields (HPF; × 1,000) from each duplicate well. Chemotactic response was defined as the mean number of migrated cells per HPF. F-12 without FCS was incubated identically with A549 cells, and the supernatant harvested were used to determine background monocyte migration. Formyl-methionyl-leucyl-phenylalanine (FMLP; 10^{-8} M in F-12, Sigma) and normal human serum that was complement activated by incubation, with *Escherichia coli* endotoxin and diluted ten-fold with F-12, were used as positive controls [10].

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 by using A549 cell supernatant harvested at 72 h in response to 500 pg·mL^{-1} of IL-α and 1,000 U·mL^{-1} of TNF-α. In order to do this, various concentrations of A549 cell supernatants (1:27, 1:9, 1:3, 1:1) were placed above the membrane with cells and below the membrane.

To ensure that monocytes and not lymphocytes, were the primary cells that migrated, some of the membranes were stained with α-naphthyl acetate esterase according to the manufacturer's directions (Sigma).

Partial characterization of MCF

Because MCF were detected in the A549 cell culture supernatants, partial characterization of MCF was performed using supernatant harvested at 72 h incubation in response to 500 pg·mL^{-1} of IL-1α and 1,000 U·mL^{-1} of TNF-α. Sensitivity to proteases was tested with trypsin treatment (final concentration 100 μg·mL^{-1}; Sigma) for 30 min at 37°C followed by the addition of 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 twice with ethyl acetate, decanting the lipid phase after each extraction, evaporating the ethyl acetate to dryness, and resuspending the extracted material in F-12 used for the cell culture before the chemotaxis assay. Heat sensitivity was determined by heating the culture supernatant at 98°C for 30 min to examine the heat stability.

Partial purification of the chemotactic factors by column chromatography

In order to determine the approximate molecular weight of the released chemotactic factors of A549 cell supernatants, which were harvested at 72 h in response to 500

pg·mL^{-1} of IL-1α and 1,000 U·mL^{-1} of TNF-α, molecular sieve column chromatography was performed using Sephadex G-100 (25 × 1.25 cm, Pharmacia, Piscataway, NJ, USA) at a flow rate of 6 mL·h^{-1}. The A549 culture supernatant was eluted with PBS, and every other fraction after the void volume was evaluated for MCF in duplicate.

Effects of metabolic and receptor determinants on MCF release

Although A549 cells were capable of releasing lipoxygenase metabolites which may account for the released MCF, the effects of nonspecific lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA; 100 μM, Sigma) and diethylcarbamazine (DEC; 1 mM, Sigma), and 5-lipoxygenase inhibitor AA-861 (100 μM, Takeda Pharmaceutical Co., Tokyo, Japan) were evaluated. The effect of protein synthesis inhibitor, cycloheximide (10 μg·mL^{-1}, Sigma), was also evaluated. 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* lipopolysaccharide and did not cause cytotoxicity to A549 cells after 72 h incubation [10, 24].

LTB_4 receptor-antagonist (ONO 4057; Ono pharmaceutical Co., Tokyo, Japan) and platelet-activating factor (PAF) antagonist (TCV 309, Takeda Pharmaceutical Co.) at concentrations of 10^{-5} M were used to evaluate the responsible chemotactic factor in the column chromatography-separated lowest molecular weight peak in IL-1α and TNF-α induced MCF.

Measurement of LTB_4 and PAF in the supernatant

The measurement of LTB_4 was performed by radioimmunoassy (RIA) [25]. Briefly, ethanol samples were centrifuged at 5,500 × *g* at 0°C. The supernatants were evaporated under N_2 gas at 37°C to cause ethanol evaporation. To each sample, 10 mL of distilled water were added. These samples were acidified to pH 4.0 with 0.1 M hydrochloric acid and applied to Sep-pak C18 columns (Waters Associates, Milford, MA, USA), the columns were washed with 10 mL of distilled water and 20 mL of petroleum ether, and then eluted with 15 mL of methanol. These eluates were dried with N_2 gas at 37°C and redissolved in 20 μL of methanol and 180 μL of RIA buffer (50 mM Tris-HCl buffer containing 0.1% (w/v) gelatin, pH 8.6). Anti-LTB_4 serum, [5, 6, 8, 9, 11, 12, 14, 15, ^{3}H (N)] LTB_4, and synthetic LTB_4 were purchased from Amersham Co. (Arlington Heights, IL, USA). [^{3}H] LTB_4 was diluted in RIA buffer and aliquots of 100 μL (containing ~66.7 Bq) were mixed with 100 μL of standards or samples in disposable siliconized tubes. Anti-LTB_4 serum diluted in RIA buffer (100 μL) and RIA buffer (100 μL) were added to give a total incubation volume of 400 μL, and the mixture was incubated at 4°C for 18 h. Free LTB_4 was adsorbed onto dextran-coated charcoal. The supernatant containing the antibody-bound LTB_4 was decanted into scintillation vials after centrifugation at 2,000 × *g* for 15 min. Scintillation fluid (Aquazol 2, NEN Co., Boston, MA, USA) was added, and radioactivity was counted by a scintillation counter (Tri-carb-3255, Tackard Co., IL, USA) for 4 min.

PAF concentration in the supernatant was measured by a scintillation proximity assay (SPA) system. 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 for measurement of LTB_4.

Measurement of MCP-1, PDGF, macrophage protein-1α, RANTES, GM-CSF, and TGF-β in the supernatants. The concentrations of MCP-1, macrophage inflammatory protein (MIP)-1α, PDGF, RANTES, GM-CSF, and TGF-β in A549 cell supernatant harvested at 72 h in response to 500 pg·mL^{-1} of IL-1α and 1,000 U·mL^{-1} of TNF-α were measured by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's directions. MIP-1α, PDGF, GM-CSF, and RANTES kits were purchased from Amersham (Buckinghamshire, UK), and the minimum concentration detected by these methods was 46.9 pg·mL^{-1} for MIP-1α, 0.31 ng·mL^{-1} for PDGF, 2.0 pg·mL^{-1} for GM-CSF, and 15.6 pg·mL^{-1} for RANTES. MCP-1 and TGF-β kits were purchased from R&D systems (Minneapolis, MN, USA), and the minimum concentration for MCP-1 and TGF-β were 31.3 pg·mL^{-1} and 0.31 ng·mL^{-1}, respectively.

Effects of polyclonal antibodies to MCP-1, PDGF, MIP-1α, GM-CSF, RANTES, and TGF-β

The neutralizing antibodies to MCP-1, RANTES, GM-CSF, PDGF, MIP-1α and TGF-β were purchased from Genzyme and were added to the A549 cell supernatants, which were harvested at 72 h in response to 500 pg·mL^{-1} of IL-1α and 1,000 U·mL^{-1} of TNF-α at the suggested concentrations to inhibit these cytokines and incubated for 30 min at 37°C. These samples were then used for a chemotactic assay. These antibodies did not influence the chemotactic response to endotoxin-activated serum (data not shown).

Statistics

Differences between groups were tested for significance using one-way analysis of variance with Duncan's multiple range test applied to data at specific time and dose points. In all cases, a p-value <0.05 was considered significant. Data in figures and tables are expressed as means±SEM.

Results

Release of MCF from A549 cell monolayers

A549 cells released MCF in response to IL-1α and TNF-α in a dose-dependent fashion ($p<0.01$, fig. 1). The lowest dose of IL-1α and TNF-α to stimulate A549 cells was 5.0 pg·mL^{-1} and 10 U·mL^{-1}, respectively. Increasing concentrations of IL-1α and TNF-α progressively increased the release of MCF. Although A549 cells released MCF in the baseline condition. The IL-1α and TNF-α stimulated release of MCF from A549 cells reached a plateau after 72 h (fig. 2). The chemotactic responses to FMLP and activated serum were 68.3±3.2 and 75.3±6.4 monocytes·HPF^{-1}, respectively.

Because IL-1α and TNF-α had intrinsic chemotactic activity for monocytes [26], the constitutive supernatant mixed with maximal doses of IL-1α (500 pg·mL^{-1}) and TNF-α (1,000 U·mL^{-1}) were tested for monocyte chemotaxis. These aliquots did not cause synergistic activity for

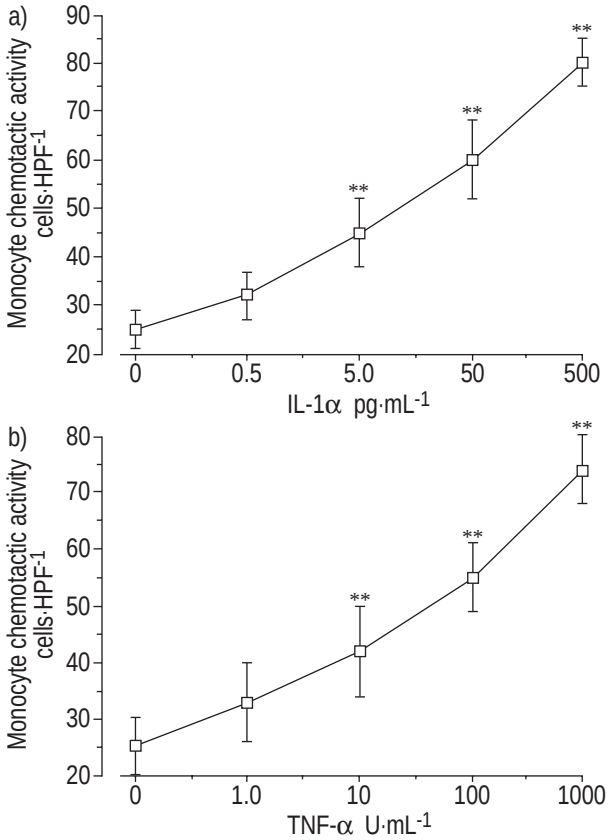

Fig. 1. – Dose-dependent release of monocyte chemotactic factors in response to interleukin (IL)-1α (a) and tumour necrosis factor (TNF)-α (b) from A549 cell monolayers after 72 h incubation (n=8). Values are expressed as means±SEM. HPF: high power field. **: p<0.01, compared with medium alone.

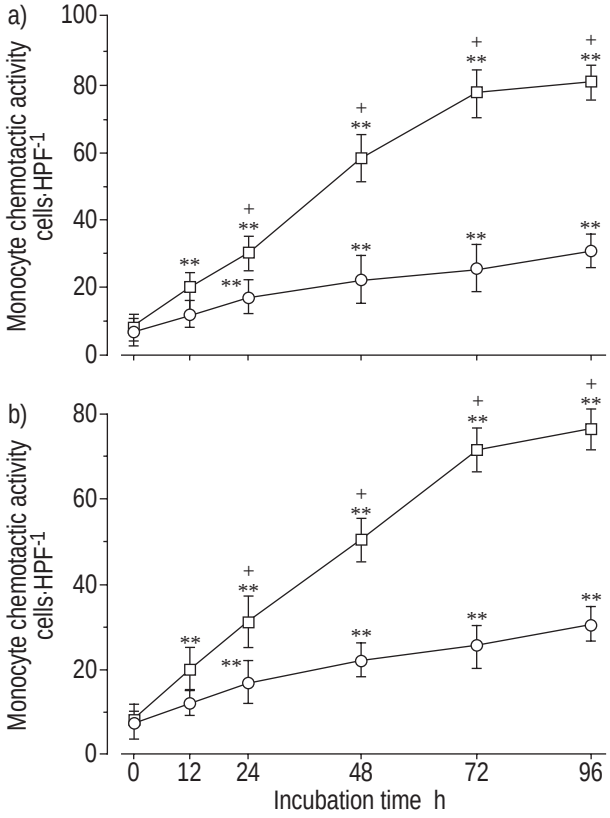

Fig. 2. – Time-related release of monocyte chemotactic factors in response to 500 pg·mL^{-1} of interleukin (IL)-1α (□; a) and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α (□; b) from A549 cell monolayers (n=8). ○: Baseline release of the monocyte chemotactic factors. Values are expressed as means±SEM. HPF: high power field. **: p<0.01, compared with supernatant without incubation; $^{+}$: p<0.01, compared with constitutive release of monocyte chemotactic factor without stimulation.

monocyte chemotaxis. Although IL-1α (50.0–5.0 pg·mL^{-1}) and TNF-α (100–10 U·mL^{-1}) both caused monocyte chemotaxis, the potential of IL-1α and TNF-α as MCF was less than one-third that of A549 cell supernatant stimulated by IL-1α and TNF-α. Thus, the observed MCF was indeed released from A549 cells.

Checkerboard analysis revealed that A549 cell supernatants stimulated by IL-1α and TNF-α induced monocyte migration in the presence of a concentration gradient across the membrane. Thus, the migration in response to IL-1α and TNF-α stimulated A549 cell supernatant was predominantly chemotactic and partially chemokinetic.

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

Partial characterization of MCF

The released MCF in response to IL-1α and TNF-α was heterogeneous in its character. MCF was sensitive to heat, digested by trypsin and partially extractable into ethyl acetate (fig. 3).

Effects of metabolic inhibitors on the release of MCF

Incubation of A549 cells with cycloheximide inhibited the release of MCF (fig. 4). The nonspecific lipoxygenase inhibitors, NDGA and DEC attenuated the release of MCF in response to IL-1α and TNF-α (p<0.01, fig. 4).

AA-861 inhibited the release of MCF (p<0.01, fig. 4). NDGA, DEC and AA-861 did not have any effects on FMLP and activated serum-induced monocyte chemotaxis (data not shown). Thus, MCF in response to IL-1α and TNF-α was, at least partly, composed of 5-lipoxygenase-derived chemotactic factors and peptides.

Partial purification of MCF

Molecular sieve column chromatography revealed that the released MCF was heterogeneous in size even in unstimulated supernatant (fig. 5a, circles). At least four peaks of activity were separated by column chromatography constitutively, with the estimated molecular weight at or near BSA (66.2 kDa), two broad peaks before cytochrome C and one small peak after cytochrome C (12.3 kDa), and an additional peak which eluted near quinacrine (450 Da). Molecular weight peaks before cytochrome C and quinacrine became prominent by the stimulation of

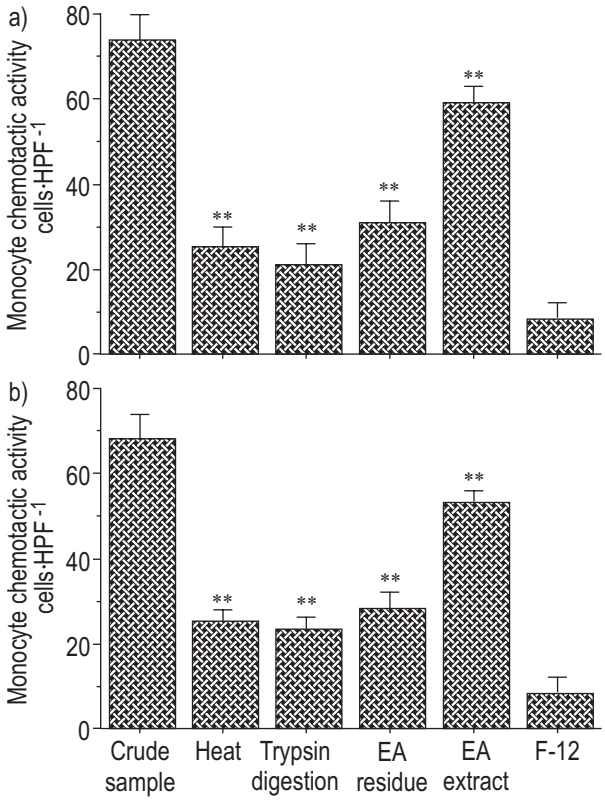

Fig. 3. – Partial characterization of the released monocyte chemotactic factors from A549 cell supernatants harvested after 72 h incubation in response to 500 pg·mL^{-1} of interleukin (IL)-1α (a) and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α (b) (n=5). EA: ethyl acetate; F-12: culture medium. Values are expressed as means±SEM. HPF: high power field. **: p<0.01, compared with crude sample.

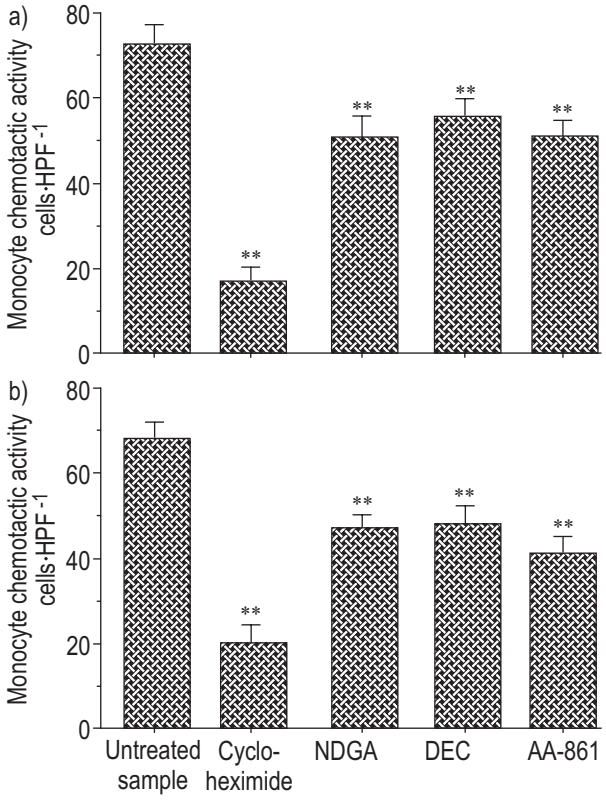

Fig. 4. – The effects of nordihydroguaiaretic acid (NDGA), diethylcarbamazine (DEC), AA-861 (5-lipoxygenase inhibitor), and cyclohexamide on the release of monocyte chemotactic factors in response to 500 pg·mL^{-1} of interleukin (IL)-1α (a) and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α (b) from A549 cell monolayers after 72 h incubation (n=5). Values are expressed as means±SEM. HPF: high power field. **: p<0.01, compared with stimulus alone.

IL-1α and TNF-α (fig. 5a and b). The addition of cycloheximide prior to IL-1α and TNF-α treatment inhibited the release of these high molecular weight MCF (data not shown).

Effects of LTB_4 and PAF receptor-antagonists on the total and the lowest molecular weight MCF

The total MCF released in response to IL-1α and TNF-α was inhibited by the addition of the LTB_4 receptor-antagonist, ONO 4057, by 50% after 24 h incubation and 30% after 72 h incubation (fig. 6), and the lowest molecular weight MCF separated by molecular sieve column chromatography was inhibited by ONO 4057 (p<0.01, table 1). The effect of the PAF receptor-antagonist, TCV 309, on MCA was not significant. Each receptor-antagonist at the concentration of 10^{-5} M completely inhibited the neutrophil migration in response to LTB_4 (10^{-7} M) and PAF (10^{-7} M), [27–29] but did not show any inhibitory effects on monocyte chemotaxis induced by FMLP and activated serum (data not shown).

Concentrations of LTB_4 and PAF in the supernatant

The concentrations of LTB_4 in the supernatant in response to IL-1α and TNF-α were 68.8±12.4 ng·mL^{-1} and 65.4±13.4 ng·mL^{-1}; these values were not significantly different from control 52.9±6.8 ng·mL^{-1} (n=6, p>0.05). These concentrations of synthetic LTB_4 induced monocyte chemotaxis (100 pg·mL^{-1} of LTB_4: 39.3±4.5 cells·HPF^{-1}; 50 pg·mL^{-1}: 30.3±3.8 cells·HPF^{-1}) [30]. However, PAF in the supernatant was not detected even in response to IL-1α and TNF-α stimulation (below 40 pg·mL^{-1}).

Concentrations of MCP-1, GM-CSF, TGF-β, and RANTES in the supernatant

The measurement of chemotactic cytokines by ELISA revealed that A549 cells released MCP-1 and TGF-β constitutively [10]. IL-1α and TNF-α stimulated the release of MCP-1, GM-CSF, RANTES, and TGF-β significantly (table 2). MIP-1α was not detected in A549 cell supernatant in response to IL-1α and TNF-α and in the control condition. PDGF was detected in two out of eight samples in the control, but IL-1α and TNF-α did not stimulate the release of PDGF in the supernatants (three out of eight IL-1α samples and four out of eight TNF-α samples were negative).

Effects of antibodies blocking GM-CSF, PDGF, MCP-1, MIP-1α, RANTES, TGF-β on MCF in the supernatants

Because A549 cells had the potential to release chemotactic cytokines, and because chemotactic cytokines produced from A549 cells might be responsible for the

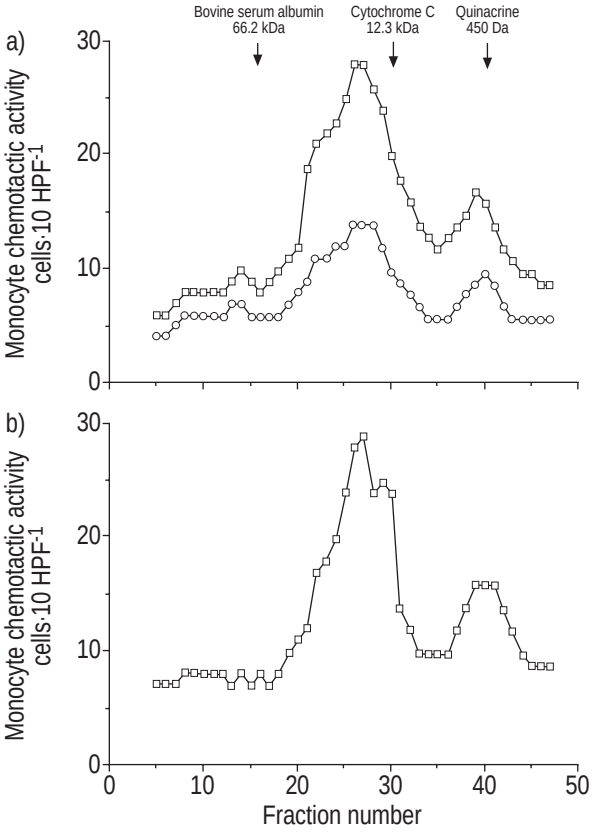

Fig. 5. – Molecular sieve column chromatographic findings of monocyte chemotactic factors of A549 cell supernatants harvested at 72 h in response to 500 pg·mL^{-1} of interleukin (IL)-1α (a) and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α (b). □: Molecular profiles of moncyte chemotactic factors in response to IL-1α and TNF-α; ○: molecular profile of the constitutive release. These data are the representative data out of four experiments.

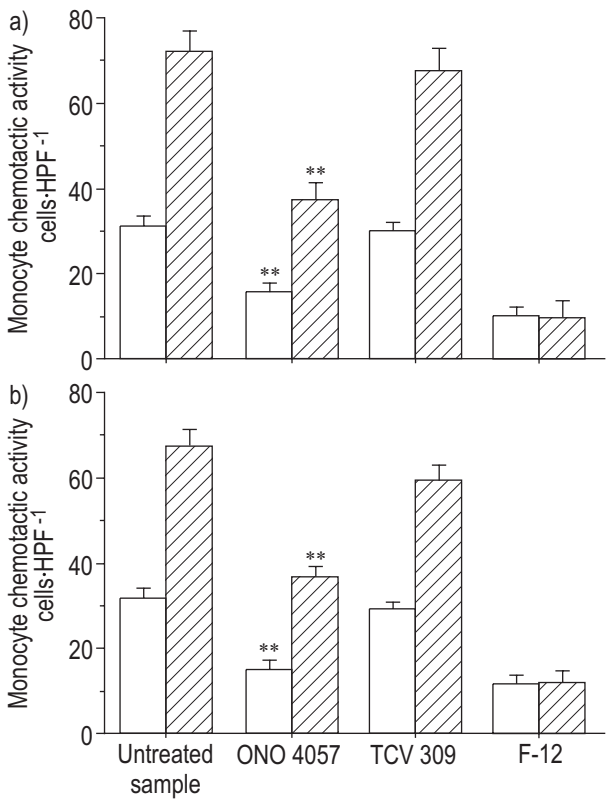

Fig. 6. – Effects of leukotriene B_4 (ONO 4057) and platelet activating factor (TCV 309) receptor-antagonists on the total monocyte chemotactic activity released from A549 cell monolayer in response to interleukin (IL)-1α (a) and tumour necrosis factor (TNF)-α (b) (n=5). □: 24 h supernatants; ▨: 72 h supernatants. Values are expressed as means±SEM. HPF: high power field. **: p<0.01, compared with untreated sample.

chemotactic activity, polyclonal blocking antibodies to GM-CSF, PDGF, MCP-1, MIP-1α, RANTES, and TGF-β were used. These antibodies inhibited the monocyte chemotactic responses to each human recombinant chemokine obtained from Genzyme (data not shown). Although MCP-1 and TGF-β antibodies inhibited MCF released constitutively [10], anti-GM-CSF, anti-MCP-1 and anti-RANTES antibodies significantly blocked MCF in response to TNF-α and IL-1α (fig. 7). The inhibitions by antibodies were almost identical in response to IL-1α and TNF-α. Anti-MCP-1, anti-GM-CSF, and anti-RANTES antibodies inhibited 40, 5, and 20% of the total MCF harvested after 24 h incubation and 50, 20, and 10% of the total MCF harvested after 72 h incubation (fig. 7). Anti-TGF-β antibody inhibited MCF in response to IL-1α, but not to TNF-α. Anti-MIP-1α and anti-PDGF antibodies did not inhibit MCF in response to either IL-1α or TNF-α stimulation. These antibodies inhibited the corresponding molecular weight chemotactic activity separated by molecular sieve column chromatography (table 3).

Discussion

The migration of monocytes from the peripheral blood to the lung interstitium and the air space is an important event both to maintain the pool of lung phagocytes [2, 3] and to respond to a variety of lung insults. Movement of inflammatory cells from the vascular space to a site of injury is dependent on the co-ordinated expression of adhesion molecules on both the monocytes and endothelium, and on the generation of a chemotactic gradient *via* the elaboration of specific chemotactic factors [31]. In the present study, the relative potential of various MCF released from A549 cells in response to TNF-α and IL-1α were evaluated. TNF-α and IL-1α induced the release of MCF in a dose- and time-dependent manner. LTB_4 receptor-antagonist inhibited the total MCF by 50% after 24 h and 30% after 72 h incubation. Although MCP-1,

Table 1. – Effects of leukotriene B_4 (ONO 4057) and platelet-activating factor (TCV 309) receptor-antagonists on the column chromatography-separated lowest molecular weight monocyte chemotactic factor (n=4)

	IL-1α	TNF-α
Crude fraction	25.7±3.5	23.4±2.5
+ ONO 4057	13.5±2.2**	11.3±2.2**
+ TCV 309	26.9±2.1	22.3±1.3
F-12 (negative control)	10.3±1.7	9.8±2.1

Values are expressed as means±SEM (cells·high power field^{-1}). IL: interleukin; TNF: tumour necrosis factor. **: p<0.01, compared with crude fraction.

Table 2. – Release of monocyte chemoattractant protein (MCP)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), "regulated on activation, normal T-cells, expressed and secreted" (RANTES), and transforming growth factor (TGF)-β from A549 cell monolayers in response to 72 h incubation with 500 pg·mL^{-1} of interleukin (IL)-1α and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α

	Control	IL-1α	TNF-α
MCP-1	2630±530	37600±4500**	36500±4800**
GM-CSF	ND	124±3**	63±3**
RANTES	ND	378±72**	1266±85**
TGF-β	482±23	931±21**	610±35**

Values are expressed as means±SEM (pg·mL^{-1}) (n=8 monolayers). ND: not detected. **: p<0.01, compared with the control.

GM-CSF, RANTES, and TGF-β were released significantly in response to TNF-α and IL-1α antibodies to MCP-1, GM-CSF, and RANTES inhibited MCF activity by 40, 5, and 20% after 24 h incubation and 50, 20, and 10% after 72 h incubation respectively. Anti-MCP-1, anti-GM-CSF, and anti-RANTES antibodies and LTB_4 receptor-antagonist inhibited the corresponding column chromatography-separated molecular weight peaks. These data indicate that A549 cells released MCP-1 and LTB_4 rather than GM-CSF, RANTES, and TGF-β as MCF, suggesting that ATII cells may modulate the monocyte recruitment in lung inflammatory responses by releasing MCF.

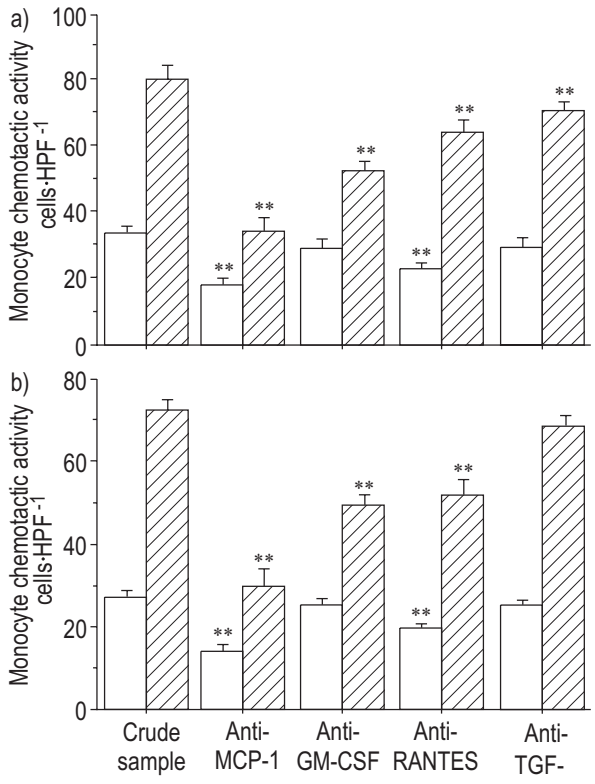

Fig. 7. – Effects of blocking antibodies on monocyte chemotactic factors of A549 cell supernatants harvested at 72 h in response to 500 pg·mL^{-1} of interleukin (IL)-1α (a) and 1,000 U·mL^{-1} of tumour necrosis factor (TNF)-α (b) (n=6). □: 24 h supernatants; ▨: 72 h supernatants. Values are expressed as means±SEM. HPF: high power fields; MCP: monocyte chemoattractant protein; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation, normal T-cells, expressed and secreted; TGF: transforming growth factor. **: p<0.01, compared with the untreated sample.

Table 3. – Effects of anti-monocyte chemoattractant protein (MCP)-1, anti-granulocyte-macrophage colony-stimulating factor (GM-CSF), and anti-regulated on activation, normal T-cells, expressed and secreted (RANTES) antibodies on the corresponding column chromatography-separated molecular weight monocyte chemotactic factor in A548 cell supernatant following incubation with interleukin (IL)-1α (500 pg·mL^{-1}) or tumour necrosis factor (TNF)-α (1,000 U·mL^{-1})

	IL-1α	TNF-α
Fraction 23	22.7±3.5	18.4±2.6
Anti-GM-CSF	14.5±2.2**	12.7±1.6**
Nonimmune IgG	23.3±1.7	19.8±2.1
Fraction 27	27.7±2.6	24.4±3.1
Anti-MCP-1	15.5±1.3**	15.3±2.4**
Fraction 32	16.9±2.1	14.3±1.3
Anti-RANTES	11.3±1.7	11.8±1.2
F-12 (negative control)	10.3±1.7	9.8±2.1

Values are expressed as means±SEM (cells·high power field^{-1}) (n=4). IgG: immunoglobulin G. **: p<0.01, compared with crude fraction.

Since A549 cells are derived from a tumour cell-line which has some characteristics of type II pneumocytes, the question remains as to whether the human type II cells respond to IL-1α and TNF-α in a similar way as A549 cells. In favour of this, it may be argued that rat ATII epithelial cells in primary culture responded in a similar manner to A549 cells to IL-1α and IL-1β and TNF-α, releasing MCP-1 and GM-CSF [20, 32]. Similarly human type II cells responded to smoke extract and neutrophil elastase as well as A549 cells [33]. These data may support the validity of using A549 cells in this context.

A549 cells have the potential to release many chemokines, including MCP-1, GM-CSF, TGF-β and RANTES. Among them, MCP-1 was a predominant MCF in the present study. RANTES, GM-CSF, and TGF-β are at least partly responsible. However, additional peaks of MCF were seen in the molecular sieve column elutions corresponding to molecular masses of approximately 65 kDa and <1 kDa. The high molecular mass fraction may represent a fibronectin fragment or other matrix degradation products [34, 35], whereas the low molecular mass fraction may represent bioactive lipid mediators. The low molecular mass appeared to be LTB_4 or other 5-lipoxygenase products, because AA-861, a specific 5-lipoxygenase inhibitor, attenuated the release of MCF and because an LTB_4 receptor-antagonist attenuated the low molecular mass chemotactic activity. Although a number of bioactive substances, including complements [36], are likely to participate in MCF from A549 cells, MCP-1 and LTB_4 appear to be the predominant MCF secreted by A549 cells rather than GM-CSF, RANTES, and TGF-β.

The LTB_4 receptor-antagonist significantly attenuated the chemotactic response in the supernatant. However, the release of LTB_4 from A549 cells in response to TNF-α and IL-1α was not significant compared with constitutive release assessed by RIA. CHAUNCEY *et al.* [37] reported that rat ATII epithelial cells have the potential to release cyclooxygenase products rather than LTB_4 in response to the

calcium ionophore, A23187 [37]. However, they observed constitutive release of LTB_4 and a small but significant increase in LTB_4 release in response to A23187 from rat ATII epithelial cells [37]. In the present study, although IL-1α and TNF-α did not remarkably augment the release of LTB_4 assessed by RIA, the concentration of constitutively released LTB_4 was high enough for MCF, as has been reported previously [10].

PAF is one of the possible candidates responsible for the chemotactic activity, and it is reported that A549 cells have the potential to release PAF in response to A23187 or phorbol myristate acetate (PMA) [38]. The PAF synthesized by A549 cells was found to be mainly associated with the cell membrane with <10% release into the medium, and the release of PAF was dependent on the presence of BSA. Because IL-1α and TNF-α stimulated the release of inflammatory cytokines, they seemed to initiate cellular signalling leading to the activation of inositol pathway and calcium release [39, 40]. However, the release of PAF was not detected by the receptor-antagonist assay and direct measurement of PAF by RIA in the supernatant. The absence of BSA and the relatively weak potency of A549 cell stimulation by IL-1α and TNF-α compared with A23187 or PMA might be responsible.

TGF-β induces monocyte chemotaxis at concentrations from 0.1–10 $pg \cdot mL^{-1}$ [41]. At higher concentrations, the chemotactic response of monocytes declined. WAKEFIELD *et al.* [42] reported that the biologically inactive form of TGF-β, which constitutes >98% of autocrine TGF-β secreted by all of 12 different cell types assayed, was shown to be unable to bind to the receptor without prior activation. Although the concentrations of TGF-β were far above the monocyte chemotactic range, *i.e.* 500–700 $pg \cdot mL^{-1}$ in the present study, the active form of TGF-β might be <2% in the A549 cell supernatant. This may account for the small potential of TGF-β as MCF.

Cellular communication between immune and nonimmune cells is likely to be an essential process in the initiation, maintenance, and resolution of an inflammatory response. In addition, networking between neighbouring cells is operative in the generation of several cytokines. The macrophage products, tumour necrosis factor and interleukin-1, are important signals for the secretion of cytokines by nonimmune cells [12, 14]. The present findings suggest that the type II pneumocytes may participate in the dynamic process of monocyte recruitment *via* the secretion of monocyte chemoattractant protein-1, leukotriene B_4, granulocyte-macrophage colony-stimulating factor, transforming growth factor-β, and "regulated on activation, normal T-cells, expressed and secreted". The secretion of monocyte chemotactic factors appears to be dependent upon cytokine networking between monocyte/macrophages and neighbouring nonimmune cells.

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