Bleomycin Stimulates Lung Fibroblasts to Release Neutrophil and Monocyte Chemotactic Activity

Akemi Takamizawa, Sekiya Koyama, Etsuro Sato, Takeshi Masubuchi, Keishi Kubo, Morie Sekiguchi, Sonoko Nagai and Takateru Izumi

*J Immunol* 1999; 162:6200-6208; ;
http://www.jimmunol.org/content/162/10/6200

**References**
This article cites 59 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/162/10/6200.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Bleomycin Stimulates Lung Fibroblasts to Release Neutrophil and Monocyte Chemotactic Activity

Akemi Takamizawa,* Sekiya Koyama,† Etsuro Sato,* Takeshi Masubuchi,* Keishi Kubo,* Morie Sekiguchi,* Sonoko Nagai,† and Takateru Izumi†

We determined whether human lung fibroblasts might release chemotactic activity for neutrophils (NCA) and monocytes (MCA) in response to bleomycin. The human lung fibroblasts supernatant fluids were evaluated for chemotactic activity by a blind well chamber technique. Human lung fibroblasts released NCA and MCA in a dose- and time-dependent manner in response to bleomycin. Checkboard analysis of supernatant fluids revealed that both NCA and MCA were chemotactic. Partial characterization revealed that NCA was partly heat labile, trypsin sensitive, and predominantly ethyl acetate extractable. In contrast, MCA was partly trypsin sensitive and ethyl acetate extractable. The release of chemotactic activity was inhibited by lipoxigenase inhibitors and cycloheximide. Molecular sieve column chromatography revealed that both NCA and MCA had multiple chemotactic peaks. NCA was inhibited by leukotriene B4 receptor antagonist and anti-IL-8 and G-CSF Abs. MCA was attenuated by leukotriene B4 receptor antagonist, and monocyte chemoattractant protein-1, GM-CSF, and TGF-β Abs. Leukotriene B4 receptor antagonist and these Abs inhibited the corresponding m.w. chemotactic activity separated by column chromatography. The concentrations of IL-8, G-CSF, monocyte chemoattractant protein-1, GM-CSF, and TGF-β in the supernatant fluids significantly increased in response to bleomycin. These data suggest that lung fibroblasts may modulate inflammatory cell recruitment into the lung by releasing NCA and MCA in response to bleomycin. The Journal of Immunology, 1999, 162: 6200–6208.

Bleomycin is one of anti-tumor antibiotics produced by Streptomyces verticillus, discovered in 1966 by Umezawa et al. (1). Although it has been used in the treatment of a variety of neoplasia, bleomycin-induced pneumonitis or pulmonary fibrosis sometimes becomes fatal (2). The incidence of bleomycin-induced pneumonitis varies from 3 to 40% (3–8), and fatalities have been reported to be 1–15% (5, 7, 9, 10) of patients who receive this agent. Bleomycin-induced pneumonitis is dose dependent and involves pulmonary inflammatory responses characterized by increases in mononuclear cells, neutrophils, fibroblast proliferation, and collagen synthesis (11, 12).

Segmentation of peripheral blood neutrophils and monocytes within the lung is a characteristic of a number of acute and chronic pulmonary diseases (13–17). The presence of neutrophils is determined by the local generation of chemotactic agents, which direct neutrophil migration from the vascular compartment to the alveolar space along chemotactic gradients. The alveolar macrophages are also derived predominantly from differentiated peripheral blood monocytes and to a limited extent from local macrophages replication (18–20). Although elicited neutrophils and macrophages serve a vital role in the host defense against a number of organisms, the presence of increased numbers of activated neutrophils and macrophages can lead to excessive tissue injury via the overzealous elaboration of inflammatory cytokines, proteolytic enzymes, and oxygen radicals (14, 21). Substantial investigation has focused on the alveolar macrophages as a primary source of chemotactic factors (22–24). However, neutrophil chemotactic activity (NCA)2 and monocyte chemotactic activity (MCA) has been found to be produced by endothelial cells (25), fibroblasts (26), and pulmonary epithelial cells (27–29).

The fibroblast is the principal cell of most connective tissues and is involved in constituting collagenous and noncollagenous components of the extracellular matrix. This synthetic activity serves an important structural function by providing a frame network for organ integrity. In addition to this traditionally accepted function, recent studies have demonstrated that fibroblasts not only serve to maintain the connective tissue but are important participants in the orchestration of acute and chronic inflammation. In this context, fibroblasts released monocyte chemoattractant protein-1 (MCP-1), GM-CSF, and TGF-β in response to IL-1, TNF-α, and platelet-derived growth factor, suggesting the contribution to certain disease states (30–36). Therefore, the fibroblast, because of its anatomic location, is in a pivotal position to participate in and direct bidirectional communications between interstitial and vascular events.

Although airway epithelial cells and alveolar macrophages may play a role in inflammatory cell migration from the interstitium to the alveolar and bronchial spaces in response to bleomycin, the underlying mechanism of inflammatory cell migration from the vascular compartment to the interstitium remains to be elucidated. The role of human lung fibroblasts (HLFs) in inflammatory cell recruitment from the vascular compartment to the interstitium in response to bleomycin is uncertain. The purpose of the present investigation is to determine whether lung fibroblasts could participate in the recruitment of inflammatory cells into the lungs. Specifically, the possibility of lung fibroblasts to release NCA and

*First Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Japan; and †Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Received for publication May 5, 1998. Accepted for publication March 1, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Address correspondence and reprint requests to Dr. Sekiya Koyama, First Department of Internal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi Matsumoto, 390 Japan.

2 Abbreviations used in this paper: NCA, neutrophil chemotactic activity; MCA, monocyte chemotactic activity; MCP, monocyte chemoattractant protein; HLF, human lung fibroblast; NDGA, nordihydroguaiaretic acid; DEC, diethylcarbamazine; LTB4, leukotriene B4.

Copyright © 1999 by The American Association of Immunologists 0022-1767/99/$02.00
Materials and Methods

Culture of HLF

We used fetal HLF (lung, diploid, human, passage 27), which is an established cell line and commercially available (American Type Tissue Culture Collection, Manassas, VA). Mononuclear cells were suspended at 1.0 × 10^6 cells/ml in F-12 supplemented penicillin (50 U/ml; Life Technologies, Grand Island, NY), streptomycin (50 μg/ml; Life Technologies), fungizone (2 μg/ml; Life Technologies), and 10% FCS (Life Technologies). HLF suspensions (3 ml) were added to a 30-mm diameter tissue culture dish (Corning, Corning, NY) and were cultured at 37°C in a 5% CO_2_ atmosphere. After 4–6 days in culture, the cells had reached confluence, and then the culture medium was replaced with 2 ml of medium supplemented as above and was incubated 1 day further.

Exposure of HLF to bleomycin

Medium was removed from cells by washing twice with serum-free F-12, and cells were incubated in the presence and absence of bleomycin. To determine the dose- and time-dependent release of NCA and MCA, the cultures were incubated at various concentrations of bleomycin (0, 0.01, 0.1, 1.0, and 10 μg/ml; Sigma, St. Louis, MO) for 12, 24, 48, 72, and 96 h at 37°C in a humidified 5% CO_2 atmosphere. Bleomycin did not cause HLF injury (no deformity of cell shape, no detachment from tissue culture dish, and ≥95% of cells were viable by trypan blue exclusion) after 72 h incubation at 10 μg/ml. However, bleomycin at 100 μg/ml caused substantial HLF cytotoxicity after 24 h incubation. The supernatants were then harvested and stored at −80°C until assayed. At least six separate HLF supernatant fluids were harvested for each experimental condition.

Measurement of NCA and MCA

Polymorphonuclear leukocytes were purified from heparinized normal human blood by the method of Boyum (37). Briefly, 15 ml of venous blood was obtained from healthy volunteers, then sedimented with 3% dextran in isotonic saline for 45 min to separate the white blood cells from RBC. The resulting cell pellet, as determined by trypan blue and erythrosin exclusion, consisted of >95% viable cells. The cells were suspended in Gey’s balanced salt solution containing 2% BSA and were cultured at 37°C in a 5% CO_2 atmosphere. After 4–6 days in culture, the cells had reached confluence, and then the culture medium was replaced with 2 ml of medium supplemented as above and was incubated 1 day further.

The chemotaxis assay was performed by a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD), as has been described previously (38). The bottom wells of the chamber were filled with 25 μl of fluid containing the chemotactic stimulus or media in duplicate. A 10-μm thick polycvinylnitrilodione-free polycarbonate filter (Nucleopore, Pleasanton, CA) with 8.0 μm for neutrophil and 4.0 μm for monocyte chemotaxis, was placed over the bottom wells. The silicon gasket and upper pieces of the chamber were applied, and 50 μl of the cell suspension was placed into the upper wells above the filter. The chambers were incubated in humidified air in 5% CO_2 at 37°C for 30 min for neutrophil chemotaxis and 90 min for monocyte chemotaxis. After incubation, the chamber was disassembled, and nonmigrated cells were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik (American Scientific Product, McGraw Park, IL), and mounted on a glass slide. Cells completely migrated through the filter were counted by using light microscopy in 10 random high power fields (magnification, ×1000) per well.

To ensure that monocytes, but not lymphocytes, were the primary cells that migrated in the monocyte chemotaxis assay, some membranes were stained with α-naphthyl acetate esterase according to the manufacturer’s directions (Sigma).

To determine whether the migration was due to movement along a concentration gradient (chemotaxis) or stimulation of random migration (chemokinesis), a checkerboard analysis was performed with HLF supernatants harvested at 72 h in response to 10 μg/ml of bleomycin (39). To do this, various dilutions of HLF supernatant fluids (1:1, 1:4, 1:16, 1:64, and 1:256) were placed below the membrane and above the membrane with target cells.

Partial characterization of NCA and MCA

Polymorphonuclear cells from healthy volunteers were stimulated with Ficoll-Hypaque density centrifugation to separate the neutrophil chemotaxis assay.

By morphology and...
LTB₄ was absorbed onto dextran-coated charcoal. The supernatant, containing the Ab-bound LTB₄, was decanted into scintillation counter following centrifugation for 15 min at 2000 × g. Scintillation fluid (Aquazol 2; New England Nuclear, Boston, MA) was added, and radioactivity was counted by a scintillation counter (Tricarb-3255; Packard, Downers Grove, IL) for 4 min.

**Effects of polyclonal Abs to IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES**

Because the results of partial characterization and the effects of metabolic inhibitors suggested the involvement of peptides as NCA and MCA, we assessed chemokines known as NCA and MCA. The neutralizing Abs to IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES (Genzyme, Cambridge, MA) were added to the HLF supernatant fluids, which were harvested at 72 h in response to 10 μg/ml of bleomycin at the suggested concentrations to inhibit these cytokines and incubated for 30 min at 37°C. Then, these samples were used for chemotactic assay. As we have previously reported that these Abs inhibited each chemokine-induced NCA and MCA, and that each Ab did not influence the neutrophil and monocyte chemotaxis induced by activated serum or FMLP (44–46). As a negative control, we used nonimmune IgG, which did not have any influences on bleomycin-conditioned medium.

**Measurement of IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES in the supernatant fluids**

The concentrations of IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES in HLF supernatant fluids cultured for 72 h at the concentration of 10 μg/ml of bleomycin were measured by ELISA according to the manufactures’ direction. GM-CSF and RANTES kits were purchased from Amersham (Buckinghamshire, England), and the minimum concentration detected by these methods was 2.00 pg/ml for GM-CSF and 15.6 pg/ml for RANTES. IL-8, MCP-1, and TGF-β kits were purchased from R & D Systems (Minneapolis, MN), and the minimum detectable concentration of IL-8, MCP-1, G-CSF, and TGF-β was 10.0 pg/ml, 31.3 pg/ml, and 0.31 ng/ml, respectively. G-CSF (chemiluminescence enzyme immunoassay method) kit was obtained from Chugai Pharmaceutical (Tokyo, Japan), and the minimum detectable concentration of G-CSF was 1.0 pg/ml.

**Evaluation of IL-8, G-CSF, MCP-1, and GM-CSF mRNA expressions**

The protein secretions of IL-8, G-CSF, MCP-1, and GM-CSF were augmented by bleomycin. RT-PCR was used to evaluate mRNA expression of IL-8, G-CSF, MCP-1, and GM-CSF in HLF in response to 10 μg/ml bleomycin after 6 h incubation. Total RNA was extracted from HLF as previously described (47). One microgram of total RNA was reverse-transcribed into cDNA with a cDNA synthesis kit (Boeringer Mannheim, Mannheim, Germany) and then amplified for 27 or more cycles in Perkin-Elmer Gene Amp PCR System 9600 (denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 30 s; Perkin-Elmer, Norwalk, CT). IL-8, G-CSF, MCP-1, and GM-CSF sense, anti-sense, and probe used in the present study were as follows: GM-CSF sense, 5'-TGA ACC TGA GTA GAG ACA CTG C-3'; anti-sense, 5'-TGA CAA GCA GAA AGT CCT TCA G-3'; probe, 5'-AGT TTT GAC CTC CAG GAG CCG ACC TGC CTA-3'; L-8 sense, 5'-AAC ATG ACT TCC AAG CTT GC-3'; anti-sense, 5'-ACT GGC ATC TTC AAT CAT GT-3'; probe, 5'-TTG AGA GTG GAC CAT CCT GGG CCA ACA CAG-3'; MCP-1 sense, 5'-TAC CAG CCA CCT TCA TTC CC-3'; anti-sense, 5'-CAG GTG TTC CAT GGA ATC CTG AA-3'; probe, 5'-GTG CAG AGG TTC GCG AGC TAT AGA A-3'; G-CSF sense, 5'-GCT TAG ACCCAA GTA GTG CAG-3'; anti-sense, 5'-AGG TGG CGT AGA ACA CGG TA-3'; probe, 5'-ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT-3'; β-actin sense, 5'-TGG GCC AGA TCA TGT TGT AG-3'; anti-sense, 5'-TCA TGA GGT AGT CAG TCA GA-3'; probe, human cDNA probe.

Preliminary studies indicated that more than 27 cycles were subsaturating for mRNA tested and thus was appropriate for comparison of relative levels of mRNA between groups. PCR products were separated by electrophoresis on 3% agarose gel and were visualized by 32P-labeled exposure. PCR band densities were determined by NIH Image (National Institutes of Health, Bethesda, MD) on unaltered, computer-scanned images. β-actin mRNA, which has been shown not to change by stimulation, was measured in both normal and stimulated RNA samples at each point, using the same cDNA that was analyzed for cytokines. Integrated OD measurements of 10 separate β-actin samples did not vary >10% from the mean integrated OD, which is an indication of expected variation resulting from experimental technique.

**Statistics**

In experiments where multiple experiments were made, differences between groups were tested for significance using one-way ANOVA with Fisher’s multiple range test applied to data at specific time and dose points. In experiments where single measurement was made, the differences between groups were tested for significance using the paired Student’s t test. In all cases, a value of p < 0.05 was considered significant. Data in figures and tables were expressed as means ± SE.

**Results**

**Release of NCA and MCA from HLF**

HLF released NCA and MCA in a dose-dependent manner in response to bleomycin (Fig. 1, A and B). The lowest doses of bleomycin to stimulate HLF were 1 µg/ml for neutrophils and 0.1 µg/ml for monocytes. Increasing concentrations of bleomycin progressively increased the release of NCA and MCA up to 10 µg/ml. At the concentration of 100 µg/ml, NCA and MCA dropped because bleomycin caused cytotoxicity to HLF after 24 h.

Although HLF released NCA and MCA constitutively, HLF further released NCA and MCA in response to bleomycin in a time-dependent manner (Fig. 2, A and B). The release of NCA was
significant after a 12-h exposure to bleomycin (Fig. 2A), and MCA was significant after 48 h (Fig. 2B). The release of chemotactic activity increased even at 96 h. Bleomycin itself did not show any chemotactic activities for neutrophils and monocytes (data not shown).

Checkerboard analysis revealed that the HLF supernatant fluids stimulated by bleomycin induced neutrophil migration in the presence of a gradient across the membrane in a concentration-dependent manner (Table I). Thus the migration of neutrophils was consistent with chemotactic rather than chemokinetic. In contrast, monocyte migration was induced slightly in the absence of gradient (Table II). Then, monocyte migration was predominantly chemotactic rather than chemokinetic.

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 morphologically 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 NCA and MCA

The NCA and MCA were heterogeneous in character. NCA was partially sensitive to heat, predominantly extracted by ethyl acetate, and partly digested by trypsin (p < 0.05; Fig. 3A). MCA was partially sensitive to heat, extracted by ethyl acetate, and predominantly digested by trypsin (p < 0.001, Fig. 3B).

Molecular sieve column chromatographic findings of the released chemotactic activities

Molecular sieve column chromatography using Sephadex G-200 revealed that NCA was heterogeneous in size (Fig. 4A). At least three peaks of NCA were separated by column chromatography with the estimated m.w. before and after cytochrome c (m.w. 12,300), as well as an additional peak that eluted after quinacrine (m.w. 450). The released MCA was also heterogeneous (Fig. 4B). At least three peaks of MCA seemed to be separated by column chromatography, with the estimated m.w. before cytochrome c, as well as an additional peak that eluted after quinacrine.

We conducted the column chromatographic separation on the heat-treated samples. As was shown in the Fig. 3, heat inactivated 60–70% of NCA and MCA. After heat inactivation, the chemotactic activity in the high m.w. disappeared, although the lowest m.w. peaks also decreased to 70%. Thus, higher m.w. materials were not a carrier for LTB4.

### Table I. Checkerboard analysis of NCA in HLF supernatant fluids

<table>
<thead>
<tr>
<th>Lower Wells</th>
<th>HBSS</th>
<th>1:256</th>
<th>1:64</th>
<th>1:16</th>
<th>1:4</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>1.0 ± 0.2</td>
<td>4.5 ± 0.5</td>
<td>3.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>1:256</td>
<td>3.0 ± 1.0</td>
<td>8.0 ± 4.0</td>
<td>8.5 ± 1.5</td>
<td>4.5 ± 0.5</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>1:64</td>
<td>9.5 ± 0.5</td>
<td>12.5 ± 2.5</td>
<td>7.0 ± 4.0</td>
<td>7.0 ± 1.0</td>
<td>2.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>1:16</td>
<td>7.0 ± 1.0</td>
<td>14.0 ± 1.0</td>
<td>8.0 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>1:4</td>
<td>40.5 ± 0.5</td>
<td>22.5 ± 2.5</td>
<td>15.5 ± 1.5</td>
<td>8.5 ± 0.5</td>
<td>5.5 ± 1.5</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>1:1</td>
<td>126.0 ± 32.0</td>
<td>61.0 ± 1.0</td>
<td>81.5 ± 5.5</td>
<td>9.0 ± 1.0</td>
<td>6.5 ± 1.5</td>
<td>4.5 ± 1.5</td>
</tr>
</tbody>
</table>

*Values are means ± SE of cells per high power field. Checkerboard analysis of HLF culture supernatant fluids harvested after 72 h in response to bleomycin at concentration of 10 μg/ml. Lower wells represent dilutions of HLF cell supernatant fluids placed in the lower wells; upper wells represent dilutions of supernatant fluids in the upper wells.*
Inhibition of the release of chemotactic activity by metabolic inhibitors

The supernatant fluids incubated with 10 μg/ml of bleomycin in the presence of NDGA, DEC, and AA-861 showed a decrease in the release of NCA and MCA \((p < 0.01; \text{Fig. 5A and B})\). Cycloheximide inhibited the release of both NCA \((p < 0.001; \text{Fig. 5A})\) and MCA \((p < 0.001; \text{Fig. 5B})\).

Inhibition of NCA and MCA by LTB₄ receptor antagonists

Both NCA and MCA of crude samples were significantly inhibited by the addition of the LTB₄ receptor antagonist, ONO 4057, by about 70% for NCA and 40% for MCA \((p < 0.01; \text{Fig. 6A and B})\). ONO 4057 also inhibited the column chromatography-separated lowest m.w. peak of NCA and MCA (about 80% for NCA and 60% for MCA). LTB₄ receptor antagonist at a concentration of \(10^{-5}\) M completely inhibited the neutrophil chemotaxis in response to \(10^{-7}\) M LTB₄ but showed no inhibitory effects on FMLP and endotoxin-activated serum-induced neutrophil and monocyte chemotaxis (data not shown).

Release of LTB₄ from HLF

The measurement of LTB₄ in the supernatant fluids by RIA revealed that HLF released significant amount of LTB₄ in the baseline culture condition. However, the addition of bleomycin at the concentration of 10 μg/ml for 72 h did not induce LTB₄ release from HLF [270 ± 20 pg/ml (control) vs 244 ± 7 pg/ml (bleomycin)].

Inhibition of NCA and MCA by polyclonal Abs to IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES

Because HLF had the potential to release chemokines, and because chemokines released from HLF might be responsible for NCA and MCA, we used polyclonal blocking Abs to IL-8, G-CSF, MCP-1, GM-CSF, TGF-β, and RANTES. Among these Abs, anti-IL-8 and G-CSF Abs inhibited NCA \((p < 0.05; \text{Fig. 7A})\), Anti-MCP-1, GM-CSF, and TGF-β Abs attenuated MCA \((p < 0.05; \text{Fig. 7B})\). In contrast, RANTES Ab did not inhibit MCA. We evaluated the effect of IL-8, G-CSF, MCP-1, GM-CSF, and TGF-β Abs on the column chromatography-separated NCA and MCA. These Abs also inhibited the chemotactic activities at the corresponding m.w. chemotactic peak about 60–80%.

We have previously reported that fibroblasts constitutively release monocyte chemokine activity (48). The released chemotactic agents were MCP-1, GM-CSF, LTB₄, and TGF-β. The proportion of chemotactic potential for MCP-1, GM-CSF, and TGF-β to attract monocytes was almost similar with bleomycin-stimulated conditioned medium. However, the proportion of LTB₄ in the total MCA became lower by the stimulation of bleomycin. In neutrophil chemotaxis, the proportion of LTB₄-induced neutrophil chemotaxis was higher than that in monocyte chemotaxis in bleomycin-stimulated medium. However, the IL-8 and G-CSF in the unstimulated fibroblasts medium explained only 5–10% of the NCA as assessed by Ab experiments (data not shown). Thus, MCP-1,

<table>
<thead>
<tr>
<th>Upper Wells</th>
<th>HBSS</th>
<th>1:256</th>
<th>1:64</th>
<th>1:16</th>
<th>1:4</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>1.0 ± 0.2</td>
<td>5.0 ± 3.0</td>
<td>3.5 ± 0.5</td>
<td>6.5 ± 2.5</td>
<td>24.0 ± 4.0</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>1:256</td>
<td>8.0 ± 3.0</td>
<td>14.5 ± 4.5</td>
<td>3.0 ± 2.0</td>
<td>7.5 ± 1.5</td>
<td>32.5 ± 2.5</td>
<td>13.0 ± 6.0</td>
</tr>
<tr>
<td>1:64</td>
<td>9.5 ± 1.5</td>
<td>13.0 ± 0.5</td>
<td>13.5 ± 3.5</td>
<td>20.0 ± 4.0</td>
<td>31.5 ± 9.5</td>
<td>36.5 ± 8.5</td>
</tr>
<tr>
<td>1:16</td>
<td>8.0 ± 3.0</td>
<td>17.5 ± 5.5</td>
<td>18.0 ± 7.0</td>
<td>46.0 ± 18.0</td>
<td>32.0 ± 8.0</td>
<td>21.5 ± 5.5</td>
</tr>
<tr>
<td>1:4</td>
<td>40.0 ± 1.0</td>
<td>33.0 ± 10.0</td>
<td>25.5 ± 5.5</td>
<td>34.0 ± 34.0</td>
<td>55.0 ± 15.0</td>
<td>37.0 ± 23.0</td>
</tr>
<tr>
<td>1:1</td>
<td>140.5 ± 2.5</td>
<td>133.0 ± 4.0</td>
<td>129.5 ± 18.5</td>
<td>89.0 ± 27.0</td>
<td>84.0 ± 20.0</td>
<td>54.0 ± 3.0</td>
</tr>
</tbody>
</table>

Table II. Checkerboard analysis of MCA in HLF supernatant fluids*
GM-CSF, TGF-β, IL-8, and G-CSF were produced from HLF by bleomycin stimulation as NCA and MCA.

The release of IL-8, MCP-1, G-CSF, GM-CSF, TGF-β, and RANTES from HLF by bleomycin

The measurement of chemotactic cytokines by ELISA revealed that bleomycin at the concentration of 10 μg/ml for 72 h incubation stimulated the release of IL-8 and G-CSF as NCA (p, 0.001; Table III) and GM-CSF, MCP-1, and TGF-β (p, 0.05; Table III). In contrast, RANTES was not detected in HLF supernatant fluids.

Augmentation of IL-8, GM-CSF, and MCP-1 mRNA expression by bleomycin

Bleomycin treatment of HLF for 6 h resulted in the augmented expression of IL-8, GM-CSF, and MCP-1 mRNA (Fig. 8). However, the expression of G-CSF was not detected under unstimulated and stimulated conditions after 40 cycles of amplification by PCR.

Discussion

The present study demonstrates that bleomycin stimulated HLF to release NCA and MCA in a dose- and time-dependent manner. Partial characterization and molecular sieve column chromatography revealed the heterogeneity of NCA and MCA. Anti-IL-8 and G-CSF Abs and LTB4 receptor antagonist inhibited NCA. Anti-MCP-1, GM-CSF, and TGF-β Abs and LTB4 receptor antagonist inhibited MCA. Although, LTB4 was released constitutively, the releases of IL-8, G-CSF, MCP-1, GM-CSF, and TGF-β were induced by bleomycin. These data suggest that an interaction between lung fibroblasts and bleomycin may modulate inflammatory cell recruitment from the vasculature to the interstitium after bleomycin exposure through the generation of chemotactic cytokines.

Fujita et al. (49) reported that the concentrations of bleomycin in blood and cancer tissue in human administrated at the dose of 15 mg/body i.v. were 0.8 μg/ml and 0.4 μg/ml. Although bleomycin

FIGURE 4. Molecular sieve column chromatographic findings of released NCA (A) and MCA (B) from HLF supernatant fluids harvested at 72 h in response to 10 μg/ml bleomycin. The data presented was representative of four experiments. NCA and MCA are on the ordinate, and fraction numbers are on the abscissa. Closed squares express with bleomycin, and closed circles express without bleomycin. The m.w. markers BSA (66,000), cytochrome c (12,300), and quinacrine (450) are indicated by arrows.

FIGURE 5. The inhibition of the release of NCA (A) and MCA (B) by 1 mM DEC, 100 μM NDGA, AA-861, and 20 μg/ml cycloheximide in response to 10 μg/ml bleomycin for 72 h incubation (n = 8). Chemotactic activity is on the ordinate, and the experimental groups are on the abscissa. Values are expressed as means ± SE. **, p < 0.01 compared with untreated supernatant fluids; #, p < 0.001 compared with untreated supernatant fluids.
is predominantly secreted from the kidney, the activity of bleomycin-inactivating enzyme (bleomycin hydrolase) is weak at skin, lung, and squamous cell carcinoma tissue. Therefore, the high concentration of bleomycin at these tissues is observed and thought to have a relationship with the tissue cytotoxicity. Thus the concentration of 0.1–1 μg/ml of bleomycin found to stimulate HLF in the present study may be relevant to the clinical concentration of bleomycin in the lung tissue.

Bleomycin is reported to stimulate T cells and alveolar macrophages, leading to pulmonary inflammatory responses characterized by an increase in leukocyte infiltration, fibroblast proliferation, and collagen synthesis. Inflammatory responses that recruit and activate large numbers of leukocytes often involve specific chemotactic mediators (50). In this context, previous studies have shown that dermal and synovial fibroblasts can release soluble chemotactic factors that direct the migration of neutrophils and monocytes in response to TNF, IL-1, and platelet-derived growth factor (30, 32, 33, 51). The present study demonstrated that HLF released NCA and MCA constitutively and further in response to bleomycin. Therefore, lung fibroblasts may modulate their local immunologic environment by releasing chemotactic activity for both neutrophils and monocytes, and may contribute to the lung inflammation in addition to T lymphocytes and alveolar macrophages in response to bleomycin.

It is reported that lung fibroblasts have the potential to release IL-8 and G-CSF in response to TNF or IL-1β (52). In the present study, the blocking Abs to IL-8 and G-CSF attenuated NCA similarly about 40%. Bleomycin significantly stimulated the release of IL-8 and G-CSF from HLF. Thus, HLF released IL-8 and G-CSF as NCA in response to bleomycin.

Wang et al. (53) reported that the concentration of G-CSF as NCA was 7–70 ng/ml. Although the concentration of G-CSF detected in the HLF supernatant fluids was less than that reported. We performed neutrophil chemotaxis by using human recombinant

---

**FIGURE 6.** The inhibition of NCA (A) and MCA (B) in the HLF supernatant fluids harvested at 72 h in response to 10 μg/ml of bleomycin by LTB4 receptor antagonist (n = 8). Chemotactic activity is on the ordinate, and the experimental groups are on the abscissa. Values are expressed as means ± SE. ***, p < 0.01 compared with crude supernatant fluids; #, p < 0.001 compared with crude supernatant fluids.

**FIGURE 7.** The effects of blocking Abs on NCA (A) and MCA (B) of HLF supernatant fluids in response to 10 μg/ml of bleomycin for 72 h incubation (n = 8). Chemotactic activity is on the ordinate, and the experimental groups are on the abscissa. Values are expressed as means ± SE. *, p < 0.05 compared with untreated supernatant fluids; **, p < 0.01 compared with untreated supernatant fluids; #, p < 0.001 compared with untreated supernatant fluids.
The extractability of NCA and MCA into ethyl acetate along with the inhibition of release by NDGA, DEC, and AA-861 suggests that the activity is composed of lipoxigenase product. The NCA and MCA was inhibited by LTB$_4$ receptor antagonist. Although, the release of LTB$_4$ from HLF in response to bleomycin was not significant compared with control, the concentration of LTB$_4$ reached the chemotactic range of neutrophils and monocytes. Thus, LTB$_4$ may be one of the important chemotactants for neutrophils and monocytes released from fibroblasts constitutively.

Although bleomycin stimulated the release of many cytokines from lung fibroblasts, it did not augment the release of LTB$_4$ by RIA. Because NCA and MCA were inhibited by LTB$_4$ receptor antagonist, and because the column chromatographic profiles showed the increase in lowest m.w., we expected the augmented release of LTB$_4$ from HLF. However, the release of LTB$_4$ was not significant. The exact mechanisms for bleomycin to stimulate fibroblasts resulting in the release of cytokines are uncertain, and the mechanism of activation or synthesis of 5-lipoxigenase in fibroblasts is also unclear. We speculate that the stimulatory potential of bleomycin is not enough for the activation or synthesis of 5-lipoxigenase in fibroblasts compared with other stimulus, which induced LTB$_4$ release from fibroblasts. However, it might be possible that bleomycin induced the release of 12- or 15-hydroxyeicosa tetraenoic acid, which were NCA and MCA, instead of LTB$_4$, and this may explain the augmentation of the lowest chemotactic peaks.

In conclusion, bleomycin stimulated HLF to release NCA and MCA. The released activities were chemotactic by checkerboard analysis. The released NCA and MCA by bleomycin were IL-8, G-CSF, MCP-1, GM-CSF, TGF-$eta$, and LTB$_4$. These results suggest that lung fibroblasts may play a role in the inflammatory cell recruitment by releasing chemotactic activity in response to bleomycin.

### References


