Bleomycin stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activities

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Bleomycin, an antineoplastic drug, is used in the treatment of a variety of tumors, the mechanisms of bleomycin-induced lung injury and fibrosis are not fully elucidated. We postulated that bleomycin might stimulate A549 cells, a type II pneumocyte cell line, to release neutrophil and monocyte chemotactic activities (NCA and MCA, respectively). To test this hypothesis, A549 cell supernatant fluids were harvested and evaluated for NCA and MCA. A549 cell supernatant fluids showed NCA and MCA in response to bleomycin in a dose- and time-dependent manner (P < 0.05). Checkerboard analysis revealed that both NCA and MCA were predominantly chemotactic. Partial characterization of the released NCA and MCA showed that the activities were partially heat labile, trypsin digested, and predominantly ethyl acetate extractable. Lipoxigenase inhibitors and cycloheximide inhibited the release of chemotactic activities significantly. Molecular-sieve column chromatography revealed that the released activities were heterogeneous. However, low-molecular-weight activity was prominent. Leukotriene B4 receptor antagonist, anti-interleukin-8, anti-granulocyte colony-stimulating factor, and anti-monocyte chemoattractant protein-1 antibodies attenuated the chemotactic activities. Immunoreactive leukotriene B4 receptor, interleukin-8, granulocyte colony-stimulating factor, and monocyte chemoattractant protein-1 significantly increased in supernatant fluids in response to bleomycin. These data demonstrate that bleomycin stimulates type II epithelial cells to release chemotactic activities and plays a role in inflammatory cell recruitment into the lung.

Type II epithelial cell; interleukin-8; monocyte chemoattractant protein-1; granulocyte colony-stimulating factor; leukotriene B4

However, the mechanisms of pulmonary fibrosis induced by bleomycin still remain to be elucidated. Especially the role of type II alveolar epithelial cells is uncertain in bleomycin-induced pulmonary fibrosis.

Inflammatory cell recruitment, which causes the lung injury, is a significant event in pulmonary fibrosis (9). In experimental models of bleomycin-induced pulmonary fibrosis as well as in human pulmonary fibrosis by therapeutically used bleomycin, the histological features include inflammatory cell recruitment, fibroblast proliferation, and collagen synthesis (7). Inflammatory cells, including neutrophils and monocytes, have the capacity to release toxic oxygen metabolites, elastolytic enzymes, and cytokines that cause lung injury.

Although alveolar epithelial cells have previously been regarded as passive bystanders in immune interactions, it is now recognized that these cells play a role in regulating the lung immune environment. Type II epithelial cells release monocyte chemoattractant activity constitutively and express interleukin (IL)-8, monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T cells expressed and secreted (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)-β in response to tumor necrosis factor (TNF)-α and IL-1 (15, 28, 29). These cytokines have the potential to attract and activate inflammatory cells, leading to the lung injury. Because inflammatory cell recruitment into the lungs plays important roles in bleomycin-induced lung injury and because type II alveolar epithelial cells participate in the lung inflammatory responses, we hypothesized that type II epithelial cells may release neutrophil (NCA) and monocyte (MCA) chemotactic activities in response to bleomycin. The results demonstrate that a human type II alveolar epithelial-like cell line (A549 cells) released NCA and MCA, including IL-8, granulocyte colony-stimulating factor (G-CSF), MCP-1, and leukotriene (LT) B4 as chemotactic factors, in response to bleomycin.

MATERIALS AND METHODS

Preparation of A549 type II alveolar epithelial cells. Because of the difficulty in obtaining primary human type II epithelial cells of sufficient purity, A549 cells (passage 75; American Type Culture Collection, Manassas, VA), a pulmonary type II epithelial cell line derived from an individual with alveolar cell carcinoma, were used (16). These cells retained many of the characteristics of normal type II epithelial cells such as surfactant production, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance (8, 28, 29). A549 cells were grown as monolayers in 100-mm tissue culture dishes. A549 cells were incubated in 100% humidity...
and 5% CO₂ at 37°C with Ham's F-12 medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml), Fungizone (2 µg/ml), and 10% heat-inactivated fetal calf serum (FCS; all from Gibco BRL, Grand Island, NY). Cells from the monolayers were harvested with trypsin (0.25%) and EDTA (0.1%) in PBS, centrifuged at low speed (250 g for 5 min), and resuspended in fresh medium at a concentration of 1.0 × 10⁶ cells/ml in 35-mm tissue culture dishes. The cells were grown to confluence on the dish for 5–7 days. After the cells reached confluence, they were used for the experiments.

Exposure of A549 cells to bleomycin. Medium was removed from the cells by washing twice with serum-free Ham's F-12 medium, and the cells were incubated in the presence and absence of bleomycin. To determine the dose-dependent release of NCA and MCA, the cultures were incubated for 12, 24, 48, and 72 h at 37°C in a humidified 5% CO₂ atmosphere at various concentrations of bleomycin (0.01, 0.1, 1.0, and 10 µg/ml; Sigma, St. Louis, MO). Bleomycin did not cause A549 cell 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 of incubation at the maximal doses. The supernatant fluids were harvested and stored at −80°C until assayed. At least six separate A549 cell supernatant fluids were harvested from the cultures for each experimental condition.

Measurement of NCA and MCA. Polymorphonuclear leukocytes were purified from heparinized normal human blood with the method of Boyum (5). Briefly, 15 ml of venous blood were obtained from healthy volunteers, then sedimented with 3% dextran in isotonic saline for 45 min to separate the white blood cells from the red blood cells. The leukocyte-rich upper layer was collected, and the neutrophils were separated from the mononuclear cells by Ficoll-Hypaque density centrifugation (Histopaque 1077, Sigma). Contaminating red blood cells were removed with a lysis solution containing 0.1% KHC₅O₃ and 0.83% NH₄Cl. The suspension was then centrifuged at 400 g for 5 min and washed three times in Hanks' balanced salt solution. The resulting cell pellet, as determined by 400 g for 5 min), and resuspended in Ham's F-12 medium used for the cell culture before the chemotaxis assay. Both extracted and extractant materials were evaluated for chemotactic activity. Heat sensitivity was determined by heating the supernatant fluids at 98°C for 15 min.

Molecular-sieve column chromatographic findings of the released chemotactic activity. To determine the approximate molecular mass of the released chemotactic activity in the supernatant fluids harvested after 72 h of incubation with 10 µg/ml of bleomycin, molecular-sieve column chromatography was performed with Sephadex G-200 (Pharmacia, Piscataway, NJ). At a flow rate of 6 ml/h, A549 cell culture supernatant fluids were eluted with PBS, and the fractions were evaluated for NCA and MCA in duplicate.

Effects of metabolic inhibitors on the release of chemotactic activity. The effects of the nonspecific lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA; 10 µM; Sigma) and diethylcarbamazine (DEC; 1 mM; Sigma) and the selective lipoxygenase inhibitor AA-861 (100 µM; Takeda Pharmaceutical, Tokyo, Japan) on the release of NCA and MCA in A549 cell supernatant fluid harvested after 72 h of incubation with 10 µg/ml of bleomycin were evaluated. To further examine the involvement of protein synthesis in the release of chemotactic activity, cycloheximide (20 µg/ml; Sigma) was added to inhibit protein synthesis (10).

Effects of LTₐ and platelet-activating factor-receptor antagonists on chemotactic activity. Because the release of NCA and MCA was blocked by 5-lipoxygenase inhibitors and because NCA and MCA were extracted into ethyl acetate, an LTₐ-receptor antagonist (ONO-4057, ONO Pharmaceutical, Tokyo, Japan) and a platelet-activating factor (PAF)-receptor antagonist (TCV-309, Takeda Pharmaceutical, Tokyo, Japan)
at a concentration of 10^{-5} M were used to evaluate the involvement of LTB4 and PAF as NCA and MCA (14, 30). These receptor antagonists at a concentration of 10^{-5} M completely blocked the neutrophil and monocyte chemotactic responses to 10^{-7} M LTB4 and PAF. But these receptor antagonists did not have any influence on the chemotactic response of neutrophils and monocytes to endotoxin-activated serum and N-formyl-methionyl-leucyl-phenylalanine (fMLP).

Measurement of LTB4 and PAF in the supernatant fluid. The concentration of LTB4 in the supernatants was measured by radioimmunoassay (RIA) as previously described (22, 23, 26). Anti-LTB4 serum, [5,6,8,9,11,12,14,15-3H(N)]-LTB4, and synthetic LTB4 were purchased from Amersham (Arlington Heights, IL). Briefly, ethanol and the supernatant mixtures were centrifuged at 5,500 g at 0°C. At a temperature of 37°C, the supernatants were evaporated under N2 gas to remove the ethanol. 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). The columns were washed with 10 ml of distilled water and 20 ml of petroleum ether, then eluted with 15 ml of methanol. These eluates were dried with N2 gas at 37°C, then redissolved in 20 μl of methanol and 180 μl of RIA buffer [50 mM Tris-HCl buffer containing 0.1% (wt/vol) gelatine, pH 8.6]. [3H]LTB4 was diluted in RIA buffer (0.1 ml containing ~4,000 dpm) and mixed with 0.1 ml of standards or samples in disposable siliconized tubes. Anti-LTB4 serum diluted with RIA buffer (0.1 ml) was added to the siliconized tubes to give a total incubation volume of 0.4 ml. The mixture was incubated at 4°C for 18 h. Free LTB4 was absorbed onto dextran-coated charcoal. The supernatant containing the antibody-bound LTB4 was decanted into a scintillation counter.
rial after centrifugation for 15 min at 2,000 g. Scintillation fluid (Aquazol 2, NEN, Boston, MA) was added, and radioactivity was counted by a scintillation counter (Tri Carb-3255, Packard) for 4 min.

PAF in the supernatant fluids was evaluated via the scintillation proximity assay system. Briefly, this assay 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 the measurement of LTB4.

Effects of polyclonal antibodies to IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β. The neutralizing antibodies to human IL-8, G-CSF, MCP-1, RANTES, GM-CSF, and TGF-β were purchased from Genzyme (Cambridge, MA). They were added at the suggested concentrations to inhibit these cytokines to the A549 cell supernatant fluids that were harvested after 72 h of incubation with 10 μg/ml of bleomycin and incubated for 30 min at 37°C. Then these samples were used for chemotactic assay. To evaluate the effect of IgG, nonimmune IgG was used as control.

Measurement of IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β in the supernatant fluids. The concentrations of IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β in A549 cell supernatant fluids cultured for 72 h at a concentration of 10 μg/ml of bleomycin were measured with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. GM-CSF and RANTES kits were purchased from Amersham, and the minimum concentrations detected by these methods were 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 concentrations of IL-8, MCP-1, and TGF-β were 10.0 pg/ml, 31.3 pg/ml, and 0.31 ng/ml, respectively. G-CSF kit was obtained from Chugai Pharmaceutical (Tokyo, Japan). The minimum detectable concentration was 1.0 pg/ml.

Statistics. In experiments where multiple experiments were done, differences between groups were tested for significance with one-way analysis of variance, with Duncan’s multiple range test applied to data at specific time and dose points. In experiments where a single measurement was made, the differences between groups were tested for significance with Student’s paired t-test. In all cases, a P value < 0.05 was considered significant. Data are expressed as means ± SE.

RESULTS

Release of NCA and MCA from A549 cells. In response to bleomycin, A549 cells released NCA and MCA in a dose-dependent manner (P < 0.05; Fig. 1). The lowest doses of bleomycin to stimulate A549 cells were 0.1 μg/ml for neutrophils and 1 μg/ml for monocytes. Increasing concentrations up to 10 μg/ml of bleomycin progressively increased the release of chemotactic activity.

A549 cells released NCA and MCA in response to bleomycin in a time-dependent manner (P < 0.05; Fig. 2). After exposure to bleomycin, the release of NCA increased significantly after 72 h and MCA increased significantly after 48 h. Both NCA and MCA were cumulative even at 72 h. Bleomycin itself did not show any chemotactic activities for neutrophils and monocytes (data not shown).

The chemotactic responses to LTB4 at a concentration of 10−7 M as a positive control were 1,020 ± 74 cells/10 HPF for neutrophils and 756 ± 34 cells/10 HPF for monocytes.

Checkerboard analysis revealed that the A549 cell supernatant fluids stimulated by bleomycin induced neutrophil and monocyte migration with increasing chemotactic activities in the presence of a gradient across the membrane (Table 1). Thus the migration of neutrophils and monocytes was predominantly consistent with chemotactic release, rather than 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 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.

| Table 1. Checkerboard analysis of A549 cell culture supernatant fluid harvested after 72 h in response to 10 μg/ml of bleomycin |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                | Lower Well     | RPMI           | 1:256          | 1:64           | 1:16           | 1:4            | 1:1            |
| Neutrophils    |                |                |                |                |                |                |                |
| RPMI           | 94 ± 12        | 61 ± 6         | 57 ± 7         | 83 ± 5         | 56 ± 7         | 56 ± 7         | 56 ± 3         |
| 1:256          | 80 ± 5         | 45 ± 4         | 56 ± 5         | 79 ± 6         | 89 ± 7         | 63 ± 7         |
| 1:64           | 89 ± 7         | 49 ± 7         | 57 ± 7         | 121 ± 11       | 79 ± 3         | 49 ± 6         |
| 1:16           | 112 ± 11       | 79 ± 14        | 57 ± 4         | 78 ± 8         | 83 ± 5         | 79 ± 7         |
| 1:4            | 94 ± 3         | 95 ± 27        | 107 ± 12       | 56 ± 6         | 105 ± 6        | 71 ± 6         |
| 1:1            | 662 ± 27       | 627 ± 56       | 678 ± 22       | 173 ± 19       | 411 ± 21       | 149 ± 12       |
| Monocytes      |                |                |                |                |                |                |                |
| RPMI           | 51 ± 7         | 20 ± 2         | 47 ± 7         | 15 ± 4         | 36 ± 5         | 5 ± 1          |
| 1:256          | 41 ± 6         | 57 ± 11        | 24 ± 4         | 16 ± 2         | 15 ± 5         | 10 ± 3         |
| 1:64           | 25 ± 2         | 28 ± 3         | 18 ± 2         | 22 ± 3         | 16 ± 4         | 8 ± 2          |
| 1:16           | 35 ± 4         | 52 ± 6         | 28 ± 2         | 18 ± 3         | 14 ± 8         | 4 ± 1          |
| 1:4            | 58 ± 3         | 135 ± 15       | 83 ± 11        | 27 ± 8         | 54 ± 19        | 14 ± 3         |
| 1:1            | 198 ± 7        | 245 ± 34       | 142 ± 11       | 89 ± 18        | 42 ± 5         | 25 ± 4         |

Values are means ± SE in cells/10-high power fields (HPF). RPMI, RPMI 1640 medium. Lower well contained dilutions of A549 cell supernatant fluids. Upper well contained dilutions of supernatant fluids with target cells.
Partial characterization of NCA and MCA. NCA and MCA were heterogeneous in their character. Both NCA and MCA were partially sensitive to heat, digested by trypsin, and predominantly extractable into ethyl acetate (Fig. 3).

Molecular-sieve column chromatographic findings of the released chemotactic activity. The released chemotactic activities obtained from A549 cells that were incubated with 10 µg/ml of bleomycin for 72 h were evaluated by molecular-sieve column chromatography with Sephadex G-200. These experiments revealed that the released NCA obtained from unstimulated cells was heterogeneous in size (Fig. 4A). At least two peaks of activity were separated by column chromatography, with the estimated molecular mass near that of cytochrome c (molecular mass 12,300 Da) and an additional peak that eluted near quinacrine (molecular mass 450 Da). When stimulated with bleomycin, these two peaks became prominent.

The MCA from unstimulated cells was also heterogeneous (Fig. 4B). At least three peaks of activity were separated by column chromatography, with two peaks between BSA and cytochrome c and an additional peak near quinacrine. When stimulated with bleomycin, each peak became prominent.
Effects of metabolic inhibitors on the release of chemotactic activity. The supernatant fluids incubated with 10 µg/ml of bleomycin in the presence of NDGA, DEC, and AA-861 showed a significant drop in the release of NCA and MCA. Cycloheximide also inhibited the release of NCA and MCA in response to bleomycin ($P < 0.05$; Fig. 5). NDGA, DEC, AA-861, and cycloheximide did not inhibit the neutrophil migratory responses to activated serum, fMLP, or A549 cell supernatant fluids harvested after 72 h of incubation with 10 µg/ml of bleomycin (data not shown).

## Effects of LTB$_4$- and PAF-receptor antagonists on chemotactic activity.

Both NCA and MCA were significantly inhibited by the addition of the LTB$_4$-receptor antagonist ONO-4057 ($\sim$50% for NCA and 40% for MCA; Fig. 6). In contrast, the PAF-receptor antagonist TCV-309 did not inhibit NCA and MCA. Each receptor antagonist at a concentration of $10^{-5}$ M inhibited neutrophil migration in response to a $10^{-7}$ M concentration of LTB$_4$ and PAF but showed no inhibitory effects...
on lipopolysaccharide-activated serum-induced neutrophil and monocyte chemotaxis (data not shown).

Effects of bleomycin on the release of LTB₄ and PAF from A549 cells. The measurement of LTB₄ by RIA revealed that A549 cells released LTB₄ in baseline culture conditions. The addition of bleomycin at a concentration of 10 µg/ml for 72 h induced a significant increase in LTB₄ release from A549 cells (P < 0.05; Fig. 7). PAF was not detected in the supernatant fluids.

Effects of polyclonal antibodies to IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β. Because A549 cells had the potential to release chemotactic cytokines and because chemotactic cytokines produced from A549 cells might be responsible for the chemotactic activity, we used polyclonal blocking antibodies to IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β. Among these antibodies, anti-IL-8 and anti-G-CSF antibodies significantly blocked NCA (Fig. 8A). Anti-MCP-1 antibody significantly blocked MCA (Fig. 8B). We evaluated the effects of IL-8, G-CSF, MCP-1, and TGF-β antibodies on the column chromatography-separated high-molecular-mass peaks of bleomycin-induced chemotactic activity. These antibodies inhibited the chemotactic activities at the corresponding molecular-mass peak (Table 2).

Anti-IL-8 and anti-G-CSF antibodies inhibited the neutrophil migratory response to recombinant human IL-8 and G-CSF completely but did not inhibit the neutrophil migratory response to activated serum and fMLP. Anti-MCP-1 antibody also inhibited the monocyte migratory response to human recombinant MCP-1 but not to fMLP and activated serum. Nonimmune IgG did not inhibit the neutrophil and monocyte migratory responses to supernatant fluid harvested after 72 h of incubation with 10 µg/ml of bleomycin.

Effects of bleomycin on the release of IL-8, G-CSF, MCP-1, GM-CSF, RANTES, and TGF-β from A549 cells. A549 cells released detectable amounts of IL-8, G-CSF, MCP-1, and TGF-β constitutively (Fig. 9). A549 cells stimulated by bleomycin at a concentration of 10 µg/ml released IL-8, G-CSF, and MCP-1 significantly compared with the unstimulated supernatants. Bleomycin did not stimulate TGF-β significantly (Fig. 9D). RANTES and GM-CSF were not detected in A549 cell supernatant fluids in both the unstimulated and stimulated supernatant states.

**DISCUSSION**

The present study demonstrated that bleomycin stimulated A549 cells to release NCA and MCA in a dose- and time-dependent manner. Partial characterization and molecular-sieve column chromatography re-

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**Fig. 7.** Release of LTB₄ in response to 10 µg/ml of BLEO from A549 cells harvested after 72 h of incubation. CONT, control. Values are means ± SE; n = 6 monolayers. *P < 0.05 compared with supernatant fluids without BLEO.

**Fig. 8.** Effects of anti-interleukin (IL)-8, anti-granulocyte colony-stimulating factor (G-CSF), anti-transforming growth factor (TGF)-β, anti-monocyte chemoattractant protein (MCP)-1, anti-released on activation normal T cells expressed and secreted (RANTES), and anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) polyclonal antibodies on released neutrophil (A) and monocyte (B) chemotactic activity in response to 10 µg/ml of BLEO from A549 cells incubated for 72 h. Values are means ± SE; n = 6 monolayers. *P < 0.05 compared with BLEO-exposed supernatant fluids.
Table 2. Effects of specific antibodies and LTB₄-receptor antagonist on column chromatography-separated neutrophil and monocyte chemotactic peaks

<table>
<thead>
<tr>
<th>Neutrophil Chemotactic Activity</th>
<th>G-CSF antibody</th>
<th>TGF-β antibody</th>
<th>MCP-1 antibody</th>
<th>LTB₄-receptor antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 19</td>
<td>13.1 ± 2.1</td>
<td>8.4 ± 1.5*</td>
<td>7.2 ± 1.1*</td>
<td>10.3 ± 2.1*</td>
</tr>
<tr>
<td>Fraction 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+G-CSF antibody</td>
<td>15.4 ± 3.4</td>
<td>7.2 ± 1.1*</td>
<td>3.3 ± 0.4</td>
<td></td>
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<tr>
<td>Fraction 29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+LTB₄-receptor antagonist</td>
<td>32.3 ± 4.1</td>
<td>12.3 ± 4.5*</td>
<td>10.3 ± 2.1*</td>
<td></td>
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<thead>
<tr>
<th>Monocyte Chemotactic Activity</th>
<th>G-CSF antibody</th>
<th>TGF-β antibody</th>
<th>MCP-1 antibody</th>
<th>LTB₄-receptor antagonist</th>
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<tr>
<td>Fraction 14</td>
<td>9.5 ± 1.3</td>
<td>7.4 ± 1.2</td>
<td>7.5 ± 2.3*</td>
<td>12.3 ± 4.5*</td>
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<tr>
<td>Fraction 18</td>
<td>16.3 ± 2.4</td>
<td>7.4 ± 1.2</td>
<td>7.5 ± 2.3*</td>
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<tr>
<td>+MCP-1 antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 29</td>
<td>25.6 ± 3.4</td>
<td>12.3 ± 4.5*</td>
<td>7.5 ± 2.3*</td>
<td></td>
</tr>
<tr>
<td>+LTB₄-receptor antagonist</td>
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Values are means ± SE in cells/HPF; n = 4 monolayers. LTB₄, leukotriene B₄; G-CSF, granulocyte colony-stimulating factor; IL-8, interleukin-8; TGF-β, transforming growth factor-β; MCP-1, monocyte chemotactic protein-1. *P < 0.01 compared with untreated samples.

Inflammatory cell recruitment is a significant event in interstitial lung diseases that leads to pulmonary fibrosis. Bleomycin-induced lung injury involves a pulmonary inflammatory response characterized by increases in mononuclear cells and granulocytes. The mechanisms by which inflammatory cells recruit to the lung remain to be elucidated. The generation of chemotactic factors that direct neutrophils and monocytes to the interstitium and alveolar space seems essential. The experimental studies have focused on immune competent cells and fibroblasts as key sources associated with bleomycin-induced lung inflammation. However, the role of alveolar type II epithelial cells has so far received little attention.

Type II alveolar epithelial cells exist in the lung as a highly differentiated phenotype with numerous specialized functions, including production of surfactants, differentiation to type I alveolar epithelium, and release of cytokines and growth factors. In the present study, we demonstrated that type II alveolar epithelial cells released NCA and MCA in response to bleomycin. The release of NCA and MCA in the supernatant fluids was significantly increased compared with that in control fluids. The released NCA and MCA were almost similar to or more than the released activity from alveolar macrophages at 10⁶ cells/ml in response to Escherichia coli lipopolysaccharide (data not shown). Thus type II epithelial cells are one of the important cell sources for the release of NCA and MCA.

The released NCA and MCA in response to bleomycin stimulation appear to be predominantly lipid extractable and of low molecular mass. The release was inhibited by lipoxygenase inhibitors. The LTB₄-receptor antagonist inhibited the chemotactic activity. The concentration of LTB₄ in the supernatant fluids increased and reached a concentration for neutrophil and monocyte chemotaxis. Thus LTB₄ is the predominant
chemotactic activity in culture supernatant fluids of A549 cells stimulated by bleomycin.

Partial characterization of NCA and MCA showed that the released activities were, in part, heat and trypsin sensitive, and the release of NCA and MCA was inhibited by cycloheximide. These data suggest that the chemotactic activities were partly composed of protein. Molecular-sieve column chromatography of NCA and MCA revealed that there were high-molecular-mass peaks of chemotactic activities in response to bleomycin. Because the released NCA and MCA were inhibited by specific antibodies to IL-8, G-CSF, and MCP-1, these cytokines were responsible for peptide chemotactic activity. The production of these cytokines by A549 cells has been reported in response to TNF-α, IL-1, and asbestos (25, 28). In the present study, IL-8, G-CSF, and MCP-1 were released from A549 cells constitutively. Bleomycin further augmented release of these cytokines from A549 cells significantly. These findings may suggest that type II alveolar epithelial cells exposed to bleomycin may have the potential to release IL-8, G-CSF, and MCP-1 as NCA and MCA.

Although NDGA at a concentration of 100 µM inhibited the release of LTB_4 almost completely, NDGA also inhibited the release of IL-8 significantly. The addition of DEC and AA-861 did not inhibit the release of IL-8. The addition of cycloheximide at 10 µg/ml completely inhibited the release of LTB_4 from A549 cells in response to bleomycin after 72 h of incubation. In these points, NDGA and cycloheximide were not specific appropriate inhibitors. However, the effects of specific receptor antagonists and antibodies and the direct measurement of LTB_4 and each cytokine may support the release of LTB_4 and cytokines from A549 cells in response to bleomycin. TGF-β is a multifunctional mediator capable of regulating cell proliferation and differentiation as well as synthesis of many components of the extracellular matrix (27). It has been associated with fibrotic processes in many organs including the lung. TGF-β can induce monocyte chemotaxis at concentrations from 0.1 to 10 pg/ml (24). In the present study, A549 cells released TGF-β constitutively. Bleomycin did not augment the release of TGF-β significantly. Although the concentration of TGF-β in the supernatant fluids was more than the chemotactic concentration for monocytes, the proteolytic processing of TGF-β is needed for the activation of TGF-β. In the present experiment, the activation of TGF-β released from A549 cells is uncertain. The activation may not be enough for monocyte chemotaxis.

In conclusion, bleomycin stimulated A549 cells to release NCA and MCA. The released activities were chemotactic by checkerboard analysis and heterogeneous in their character. NCA and MCA included LTB_4, IL-8, G-CSF, and MCP-1. These results suggest that bleomycin may play a role in inflammatory cell recruitment by stimulating lung epithelial cells to release NCA and MCA.

**REFERENCES**


