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

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Sato, Etsuro, Sekiya Koyama, Takeshi Masubuchi, Akemi Takamizawa, Keishi Kubo, Sonoko Nagai, and Takateru Izumi. Bleomycin stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activities. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L941–L950, 1999.—Although 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. Lipooxygenase 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 (IL)-8, anti-granulocyte colony-stimulating factor, and anti-monocyte chemotactic protein-1 antibodies attenuated the chemotactic activities. Immunoreactive leukotriene B4 receptor, interleukin-8, granulocyte colony-stimulating factor, and monocyte chemotactic 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.

Bleomycin, an anticancer agent, sometimes causes diffuse interstitial lung fibrosis in humans. Therefore, clinical usage of bleomycin is limited by this life-threatening disease. The incidence of pulmonary fibrosis varies from 3 to 40% (1, 2, 12, 17, 31, 32) and fatality is 1–15% (4, 6) of patients who received this agent. Because bleomycin-induced pulmonary fibrosis in animals resembles human idiopathic pulmonary fibrosis histopathologically, bleomycin has been used to induce pulmonary fibrosis in laboratory animals (13, 21). 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 an important role 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

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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 two times 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, and 48 h at 37°C in a humidified 5% CO₂ atmosphere and the absence of bleomycin. To determine the dose-dependent release from the cells, the supernatant fluids were harvested 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 fluid samples 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% KCl 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 trypan blue and erythrosin exclusion, consisted of >96% neutrophils and >98% viable cells. The cells were suspended in Gey's balanced salt solution (GIBCO BRL) containing 2% bovine serum albumin (BSA; Sigma) at pH 7.2 to give a final concentration of 3.0 × 10⁶ cells/ml. This suspension was used for the neutrophil chemotaxis assay.

Mononuclear cells for the chemotaxis assay were obtained from normal human volunteers and were separated from the red blood cells and neutrophils by Ficoll-I Hypaque density centrifugation. The mononuclear cells were harvested at the interface. The suspension was then centrifuged at 400 g for 10 min and washed three times in Hanks' balanced salt solution. The preparation routinely consisted of 30% large monocytes and 70% small 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 × 10⁶ cells/ml. The suspension was then used for the monocyte chemotaxis assay.

The chemotaxis assay was performed in a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD) as previously described (11). The bottom wells of the chamber were filled with 25 µl of fluid containing the chemotactic stimulus or medium in duplicate. A 10-µm-thick polycarbonate filter with a pore size of 3 µm for neutrophil chemotaxis and 5 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 were placed in the upper wells above the filter. The chambers were incubated in humidified air in 5% CO₂ at 37°C for 30 min for neutrophils and 90 min for monocytes. Nonmigrated cells were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik, and mounted on a glass slide. Cells that completely migrated through the filter were counted with light microscopy in 10 random high-power fields (HPF; ×1,000) per well. To ensure that monocytes, but not lymphocytes, were the primary cells that migrated in the monocyte chemotaxis assays, some membranes were stained with α-naphthyl acetate esterase according to the manufacturer's instructions (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 A549 cell supernatant fluids harvested after 72 h of incubation with 10 µg/ml of bleomycin (34). To do this, various dilutions of A549 cell supernatant fluids (1:1, 1:2, 1:4, 1:8, and 1:16) were placed below the membrane and above the membrane with target cells. Thus neutrophil and monocyte migrations were tested by a variety of concentration gradients of supernatant fluids across the membrane. Partial characterization of NCA and MCA. Partial characterization of NCA and MCA released from A549 cells was performed with supernatant fluids harvested after 72 h of incubation with bleomycin at a concentration level of 10 µg/ml. Sensitivity to proteases was tested by incubating the supernatant fluids with trypsin (100 µg/ml; Sigma) for 30 min at 37°C followed by the addition of a 1.5 Molar excess of soybean trypsin inhibitor to terminate the proteolytic activity, and then the chemotactic activity was evaluated. The lipid solubility was evaluated by mixing the supernatant fluids two times with ethyl acetate, decanting the lipid phase after each extraction, evaporating the ethyl acetate to dryness, and resuspending the extracted material in the 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 5-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 LTβ₄ 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 LTβ₄-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 LTB
4 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.

Fig. 1. Dose-dependent release of neutrophil (A) and monocyte (B) chemotactic activities in response to bleomycin after 72 h of incubation. Values are means ± SE; n = 8 monolayers. *P < 0.05 compared with supernatant fluids without bleomycin.

Fig. 2. Time-related release of neutrophil (A) and monocyte (B) chemotactic activities in response to 10 µg/ml of bleomycin. (With bleomycin stimulation: ■, without bleomycin stimulation: ○). Values are means ± SE; n = 8 monolayers. *P < 0.05 compared with 12-h supernatant fluids. **P < 0.05 compared with supernatant fluids without bleomycin.
vial 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 LTβ.

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 concentrations in the presence of a gradient across the membrane (Table 1). Thus the migration of neutrophils and monocytes was predominantly consistent with chemotactic 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 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.

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Table 1. Checkerboard analysis of A549 cell culture supernatant fluid harvested after 72 h in response to 10 µg/ml of bleomycin

<table>
<thead>
<tr>
<th>Lower Well</th>
<th>RPMI</th>
<th>1:256</th>
<th>1:64</th>
<th>1:16</th>
<th>1:4</th>
<th>1:1</th>
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<tr>
<td>RPMI</td>
<td>94 ± 12</td>
<td>61 ± 6</td>
<td>57 ± 7</td>
<td>83 ± 5</td>
<td>56 ± 7</td>
<td>56 ± 7</td>
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<td>1:256</td>
<td>77 ± 5</td>
<td>45 ± 4</td>
<td>56 ± 5</td>
<td>79 ± 6</td>
<td>89 ± 7</td>
<td>63 ± 7</td>
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<td>1:64</td>
<td>49 ± 7</td>
<td>79 ± 14</td>
<td>67 ± 7</td>
<td>121 ± 11</td>
<td>79 ± 3</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>1:16</td>
<td>662 ± 27</td>
<td>627 ± 56</td>
<td>107 ± 12</td>
<td>56 ± 6</td>
<td>105 ± 6</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>1:4</td>
<td>660 ± 27</td>
<td>627 ± 56</td>
<td>173 ± 19</td>
<td>411 ± 21</td>
<td>149 ± 12</td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>74 ± 3</td>
<td>95 ± 27</td>
<td>173 ± 19</td>
<td>411 ± 21</td>
<td>149 ± 12</td>
<td></td>
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<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>Monocytes</th>
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<tbody>
<tr>
<td>RPMI</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>1:256</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>1:64</td>
<td>25 ± 2</td>
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<tr>
<td>1:16</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>1:4</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>1:1</td>
<td>198 ± 7</td>
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</table>

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 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 LTB₄- and PAF-receptor antagonists on chemotactic activity. Both NCA and MCA were significantly inhibited by the addition of the LTB₄-receptor antagonist ONO-4057 (~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 LTB₄ and PAF but showed no inhibitory effects.
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-
Table 2. Effects of specific antibodies and LTB₄-receptor antagonist on column chromatography-separated neutrophil and monocyte chemotactic peaks

<table>
<thead>
<tr>
<th></th>
<th>Neutrophil Chemotactic Activity</th>
<th>Monocyte Chemotactic Activity</th>
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<tbody>
<tr>
<td>Fraction 19</td>
<td>13.1 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>+ G-CSF antibody</td>
<td>8.4 ± 1.5*</td>
<td></td>
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<tr>
<td>Fraction 21</td>
<td>15.4 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>+ IL-8 antibody</td>
<td>7.2 ± 1.1*</td>
<td></td>
</tr>
<tr>
<td>Fraction 29</td>
<td>33.2 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>+ LTB₄-receptor antagonist</td>
<td>10.3 ± 2.1*</td>
<td></td>
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</tbody>
</table>

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 chemoattractant protein-1. *P < 0.01 compared with untreated samples.

revealed the heterogeneity of NCA and MCA. Anti-IL-8 and anti-G-CSF antibodies and the LTB₄-receptor antagonist inhibited NCA. Anti-MCP-1 antibody and the LTB₄-receptor antagonist inhibited MCA. Although A549 cells released IL-8, G-CSF, MCP-1, and LTB₄ constitutively, bleomycin significantly stimulated the release of IL-8, G-CSF, MCP-1, and LTB₄. These data suggest that an interaction between alveolar epithelial cells and bleomycin may modulate inflammatory cell recruitment to the alveolar space.

Because A549 cells are a cell line derived from an adenocarcinoma of the lung, release and gene regulation may be particularly upregulated. However, two reports (19, 33) described that the human primary type II alveolar epithelial cells and human primary bronchial epithelial cells released IL-8 in response to smoke extract. In these reports, the primary type II alveolar epithelial cells and bronchial epithelial cells responded to smoke extract in a manner similar to A549 cells (18). Furthermore, rat type II alveolar epithelial cells in primary culture responded in a manner similar to A549 cells to IL-1 and TNF, releasing MCP-1 and GM-CSF (3, 20). The releasing potential of MCP-1 and GM-CSF from rat primary alveolar type II cells, the major MCA in the present study, was very similar to the A549 cell response to IL-1 and TNF (unpublished observations). Then we speculated that A549 cells responded similarly to IL-1 and TNF as did rat primary type II alveolar epithelial cells. Thus the human and rat primary alveolar type II epithelial cells as well as the A549 cells were highly upregulated for the production of cytokines.

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 cycloheximide (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 LTB4 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 0.1 µg/ml completely inhibited the release of IL-8 and slightly but significantly inhibited the release of LTB4 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 LTB4 and each cytokine may support the release of LTB4 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 µg/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 LTB4, 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.

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