Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity

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Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L970–L979, 1998.—Neurological transmitters including ACh, substance P (SP), and calcitonin gene-related peptide (CGRP) play an important role in regulating airway tone, and increased bronchial reactivity to cholinergic stimulation is a well-recognized phenomenon in patients with bronchial asthma. We postulated that ACh, SP, and CGRP might stimulate alveolar macrophages (AMs) to release neutrophil, monocyte, and eosinophil chemotactic activities. To test this hypothesis, bovine AMs were isolated by bronchoalveolar lavage and cultured. AMs released chemotactic activities in response to ACh in a dose- and time-dependent manner (P < 0.05). However, SP and CGRP did not stimulate bovine AMs. Checkerboard analysis revealed that these released activities were predominantly chemotactic. Partial characterization and molecular-sieve column chromatography revealed that low-molecular-weight lipid-soluble activity was predominant. Lipooxygenase inhibitors significantly blocked the release of chemotactic activities (P < 0.05). Leukotriene B4- and platelet-activating factor-receptor antagonists blocked the chemotactic activities. Immunoreactive leukotriene B4 significantly increased in supernatant fluids in response to ACh (P < 0.05), but platelet-activating factor did not. The receptor responsible for the release of the chemotactic activities was the muscarinic M3 receptor. These data demonstrate that ACh stimulates AMs to release lipooxygenase-derived chemotactic activities and plays a role in inflammatory cell recruitment into the airway.

leukotriene B4; platelet-activating factor; calcitonin gene-related peptide; substance P

INFLAMMATORY CELLS have been implicated in injurious processes associated with both acute and chronic pulmonary disorders (17). During an attack of asthma, eosinophils accumulate in the bronchial wall as well as in the airway lumen. The presence of neutrophils and eosinophils in bronchoalveolar lavage (BAL) fluid (BALF) during a period of increased airway reactivity supports relationships among the roles of neutrophils and eosinophils in the production of the late asthmatic response and the subsequent increase in airway reactivity (22, 28). However, the mechanisms accounting for the recruitment of inflammatory cells to the airway are still unknown. The elucidation of mechanisms regulating the inflammatory cell traffic from blood vessels to airways has pathophysiological and therapeutic implications.

Many neuropeptides, including ACh, substance P (SP), and calcitonin gene-related peptide (CGRP), are localized to sensory, parasympathetic, and sympathetic neurons in the airways (5, 18). These peptides have potent effects on bronchomotor tone, airway secretions, bronchial circulation, and immune cells (12, 34). Neuropeptide inflammation can be reduced by depletion of neuropeptides from nerve endings with capsaicin or by treatment with SP and CGRP antagonists. Thus these neuronal mediators participate in neurogenic inflammation, and these antagonists have the therapeutic potential for the treatment of asthma (9, 16, 23). Furthermore, the effects of anticholinergic drugs on airways have been investigated, and a number of studies (11, 24, 41) have demonstrated their efficacy in chronic obstructive pulmonary disease (COPD) and asthma. However, the mechanisms of anticholinergic agents are less certain.

Because alveolar macrophages (AMs) are the predominant immune cells found within the air space under homeostatic conditions and can interact with other cells and molecules through the release of numerous secretory products and the expression of several surface receptors, we postulated that AMs might release neutrophil, monocyte, and eosinophil chemotactic activities (NCA, MCA, and ECA, respectively) in response to neurological transmitters including ACh, SP, and CGRP. The results demonstrated that ACh stimulated AMs to release lipooxygenase-derived NCA, MCA, and ECA. However, SP and CGRP did not stimulate AMs. The addition of a muscarinic M3-receptor antagonist attenuated the release of NCA, MCA, and ECA in response to ACh. These data may suggest the potential of ACh to stimulate AMs, resulting in the release of chemotactic activity for inflammatory cells.

MATERIALS AND METHODS

Preparation of AM cultures. AMs were recovered by BAL of bovine lungs. Briefly, bovine lungs and trachea were washed three times with 1,000 ml of sterile, pyrogen-free 0.89% NaCl. AMs recovered by BAL were filtered through a single layer of gauze, centrifuged at 400 g for 5 min, washed twice in Hank’s balanced salt solution (HBSS; Biofluids, Rockville, MD), and then resuspended at 1 × 108 cells/ml in RPMI 1640. Cell viability, determined by trypan blue exclusion, was 98% on average. Total cell numbers were counted by a hemocytometer, and differential cell counts were determined by a cytocentrifuge preparation stained with Diff-Quik. With the use of this technique, >96% of the cells recovered were macrophages, and the total cell number recovered was ~1.00 × 108 cells.

Exposure of cells to ACh, SP, and CGRP. The AMs were incubated at 1 × 106 cells/ml. To determine the dose-dependent release of NCA, MCA, and ECA, the cultures were incubated for 4 h at 37°C in a humidified 5% CO2 atmosphere with various concentrations of ACh, SP, and CGRP (0, 0.1, 1.0, 3.0, 10.0 μM). The AMs were then harvested and subjected to the appropriate chemotactic assays.

Response to ACh in a dose- and time-dependent manner (lavage and cultured. AMs released chemotactic activities in response to ACh in a dose- and time-dependent manner (P < 0.05). However, SP and CGRP did not stimulate bovine AMs. Checkerboard analysis revealed that these released activities were predominantly chemotactic. Partial characterization and molecular-sieve column chromatography revealed that low-molecular-weight lipid-soluble activity was predominant. Lipooxygenase inhibitors significantly blocked the release of chemotactic activities (P < 0.05). Leukotriene B4- and platelet-activating factor-receptor antagonists blocked the chemotactic activities. Immunoreactive leukotriene B4 significantly increased in supernatant fluids in response to ACh (P < 0.05), but platelet-activating factor did not. The receptor responsible for the release of the chemotactic activities was the muscarinic M3 receptor. These data demonstrate that ACh stimulates AMs to release lipooxygenase-derived chemotactic activities and plays a role in inflammatory cell recruitment into the airway.
10, and 100 μM; Sigma, St. Louis, MO). The time-dependent release of chemotactic activities was evaluated by cultivating AMs for 0.5, 1, 2, 4, and 6 h in the presence and absence of ACh, SP, and CGRP. In preliminary experiments, unstimulated AMs released NCA significantly, and the release of NCA reached a plateau after 8 h.

Measurement of NCA, MCA, and ECA. Polymorphonuclear leukocytes were purified from heparinized normal human blood by the method of Boyum (6). Briefly, 15 ml of venous blood were obtained from healthy volunteers and were 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 lysing solution containing 0.1% KHCO3 and 0.83% NH4Cl. The suspension was then centrifuged at 400 g for 5 min and washed three times in HBSS. The resulting cell pellet was then used for the neutrophil chemotaxis assay.

Mononuclear cells for the chemotaxis assay were obtained from normal human volunteers. With the use of Ficoll-Hypaque density centrifugation, the red blood cells and neutrophils were separated from the mononuclear cells. The mononuclear cells were harvested at the interface. The suspension was then centrifuged at 400 g for 10 min and washed three times in HBSS. The preparation routinely consisted of 30% large monocytes and 70% small lymphocytes as determined by morphology and α-naphthyl acetate esterase staining (Sigma), with >95% viability as assessed by trypan blue and erythroin exclusion. The cells were suspended in Gey’s balanced salt solution (Gibco BRL, Grand Island, NY) containing 2% BSA (Sigma) at pH 7.2 to give a final concentration of 3.0 × 106 cells/ml. This suspension was then used for the neutrophil chemotaxis assay.

Eosinophils were isolated with a modified method of Hansen et al. (14) with a magnetic cell separation system (Becton Dickinson). Briefly, venous blood anticoagulated with 130 mM trisodium citrate was obtained from normal human volunteers and diluted with PBS in a 1:1 ratio. Diluted blood was overlayed on an isotonic Percoll solution (density 1.082 g/ml; Sigma), then centrifuged at 1,000 g for 30 min at 4°C with a Beckman TJ-6 centrifuge. The supernatant and mononuclear cells at the interface were carefully removed, and red blood cells in the sediment were lysed with two cycles of hypotonic water lysis. Isolated granulocytes were washed two times with PIPES buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, and 5.4 mM glucose, pH 7.4) containing 1% defined calf serum (DCC; Hyclone Laboratories, Logan, UT), and an approximately equal volume of anti-CD16 antibody conjugated with magnetic particles (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the cell pellet. After 60 min of incubation on ice, 5 ml of PIPES buffer with 1% DCS were added to the cell-antibody mixture. The resuspended cells were washed onto the separation column positioned in the magnetic cell separation system with a strong magnetic field. The cells were eluted three times with 5 ml of PIPES buffer with 1% DCS. Purity of the eosinophils counted by Randox’s stain was >95%; viability was >99%. Purified eosinophils were washed twice in PIPES buffer with 1% DCS. The eosinophils were resuspended in Gey’s solution at 2.0 × 106 cells/ml and used for the chemotaxis assay.

Chemotaxis was assayed in a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD) as previously described (15). The bottom wells of the chamber were filled with 25 μl of fluid containing the chemotactic stimulus or medium in duplicate. Polyvinylpyrrolidone-free polycarbonate filters (10 μm thick), with pore sizes of 3 μm for neutrophil chemotaxis and 5 μm for monocyte and eosinophil chemotaxis, were 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 into the upper wells above the filter. The chambers were incubated in humidified air with 5% CO2 at 37°C for 30 min for neutrophils, 90 min for monocytes, and 180 min for eosinophils. 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 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 AM supernatant fluids stimulated with 100 μM ACh for 4 h (42). To do this, various dilutions of AM supernatant fluids (1:1, 1:4, 1:16, 1:64, and 1:256) were placed with the target cells below and above the membrane.

Effects of metabolic and receptor determinants on the release of chemotactic activity. The effects of the lipoygenase inhibitors nordihydroguaiaretic acid (NDGA; 50 μM; Sigma) and diethylcarbamazine (1 mM; Sigma) and the 5-lipoxygenase inhibitor AA-861 (100 μM, Takeda Pharmaceutical, Tokyo, Japan) on the release of NCA, MCA, and ECA were evaluated (1, 26). To further examine the involvement of protein synthesis in the release of chemotactic activity, cycloheximide (20 μM; Sigma) was added to inhibit protein synthesis (10).

To determine the receptor responsible for the release of chemotactic activity, the nicotinic-receptor antagonist p-tubocurarine (300 μM) was evaluated. The muscarinic-receptor antagonists pirenzepine (300 μM; Sigma), gallamine (300 μM; Sigma), and 4-diphenylacetoxy-N-methylpiperide methiodide (300 μM; Sigma) were also evaluated to determine the responsible muscarinic receptor (7, 13). The AMs were pretreated with these agents 30 min before the addition of ACh.

Partial characterization of the released chemotactic activity. Partial characterization of NCA, MCA, and ECA released from bovine AMs was performed with supernatant fluids harvested after 4 h of incubation with ACh at a concentration of 100 μM. 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 M 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 fluid two times with ethyl acetate, decanting the lipid phase after each extraction, evaporating the ethyl acetate to dryness, and resuspending the extracted material in RPMI 1640. Both extracted and extractant materials were evaluated for chemotactic activity. Heat sensitivity was determined by maintaining the supernatant fluids at a temperature of 98°C for 15 min.
Molecular-sieve column chromatography of the released chemotactic activity. To determine the approximate molecular weight of the released chemotactic activity in response to ACh from bovine AMs, molecular-sieve column chromatography was performed with Sephadex G-200 (Pharmacia, Piscataway, NJ). At a flow rate of 6 ml/h, AM culture supernatant fluids obtained from bovine AMs that were incubated with 100 µM ACh for 4 h were eluted with PBS, and the fractions were evaluated for NCA, MCA, and ECA in duplicate.

Results of leukotriene B₄, platelet-activating factor, leukotriene C₄, and leukotriene D₄ antagonists on chemotactic activity. To evaluate the responsible chemotactic activity, a leukotriene (LT) B₄-receptor antagonist (ONO-4057, ONO Pharmaceutical), each at a concentration of 10⁻⁵ M, were used (19, 20, 35, 36).

Measurement of LTB₄ and PAF in the supernatant fluid. The concentration of LT B₄ in the supernatant was measured by RIA as previously described (30, 32). Anti-LTB₄ serum, [5,6,8,9,11,12,14,15,−H(N)]:LT B₄, and synthetic LT B₄ were purchased from Amersham (Arlington Heights, IL). Briefly, ethanol-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 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 a mixture of 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) gelatin, pH 8.6). [3H]LTB₄ diluted in RIA buffer (0.1 ml, containing ~4,000 dpm) was mixed with 0.1 ml of standard or sample in 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 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 (Tricarb-3255, Packard) for 4 min.

PAF in the supernatant fluid 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 that in the methods of measurement of LTB₄.

Effects of anti-human interleukin-3, interleukin-5, interleukin-8, and granulocyte-macrophage colony-stimulating factor antibodies on NCA, MCA, and ECA. Interleukins (ILs) have potent chemotactic activities for neutrophils, monocytes, and eosinophils. Because bovine ILs did not cross-react with human ILs, we harvested the supernatants from human AM cultures from five healthy volunteers and five patients with interstitial lung disease in response to 100 µM ACh after 4 h of incubation.

Anti-human IL-3 (10 µg/ml), IL-5 (10 µg/ml), IL-8 (10 µg/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 µg/ml) mouse polyclonal antibodies (Genzyme, Cambridge, MA) were employed to evaluate the responsible NCA, MCA, and ECA. Nonimmune mouse IgG (Genzyme) was used as the control antibody.

Statistics. In experiments where multiple measurements were made, differences among 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.
Effects of metabolic inhibitors on the release of chemotactic activity. The supernatant fluids incubated with 100 µM ACh in the presence of NDGA, diethylcarbamazine, and AA-861 showed significant decreases in the release of NCA, MCA, and ECA (P < 0.05; Fig. 5). NCA was almost completely inhibited by NDGA compared with MCA and ECA. Cycloheximide
did not inhibit the release of NCA. In contrast, cycloheximide partially inhibited the release of MCA and ECA.

Effects of LTB4, PAF, LTC4, and LTD4 antagonists on chemotactic activity. Each chemotactic activity was significantly inhibited by the addition of the LTB4-receptor antagonist ONO-4057. Its inhibition percentages were 50% for NCA and ECA and 60% for MCA (Fig. 6). The inhibitory effects of the PAF-receptor antagonist TCV-309 on NCA, MCA, and ECA were 30% of the total chemotactic activity. Each receptor antagonist at a concentration of 10^{-5} M completely inhibited the neutrophil migration in response to a 10^{-7} M concentration of LTB4 and PAF, respectively, but showed no inhibitory effects on activated serum-induced neutrophil chemotaxis (data not shown). The LTC4- and LTD4-receptor antagonist ONO-1078 did not inhibit chemotactic activity significantly.

Effects of anti-human IL-3, IL-5, IL-8, and GM-CSF antibodies on NCA, MCA, and ECA. Although the IL-8 antibody did not influence NCA in the supernatant fluids obtained from normal human AMs either unstimulated or stimulated with ACh, the IL-8 antibody blocked NCA of the supernatant fluids obtained from patients with interstitial lung disease in both unstimulated and ACh-stimulated AMs (Table 2). The data suggested that ACh further stimulated the release of IL-8 from AMs in patients with interstitial lung disease. IL-3, IL-5, and GM-CSF antibodies did not attenuate MCA or ECA of the supernatant fluids from all volunteers (data not shown).

Table 1. Checkerboard analysis of alveolar macrophage supernatant fluids

<table>
<thead>
<tr>
<th>Lower Well</th>
<th>RPMI 1:256</th>
<th>1:64</th>
<th>1:16</th>
<th>1:4</th>
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<tbody>
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<td>166±24</td>
<td>111±12</td>
<td>154±8</td>
<td>164±7</td>
</tr>
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<td>201±24</td>
<td>211±24</td>
<td>102±14</td>
<td>224±13</td>
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<td>986±64</td>
<td>1,210±110</td>
<td>1,254±50</td>
<td>455±24</td>
<td>785±41</td>
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<td>Monocytes</td>
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<td>15±2</td>
<td>11±3</td>
<td>39±5</td>
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Values are means ± SE in no. of cells/10 high-power fields. RPMI, RPMI 1640 medium. Ratio values, dilution of alveolar macrophage culture supernatant fluid. Alveolar macrophage culture supernatant fluids were harvested after 4-h incubation with 100 µM ACh.

Fig. 3. Partial characterization of released neutrophil (A), monocyte (B), and eosinophil (C) chemotactic activities from bovine AMs (n = 8) in response to 100 µM ACh. EA, ethyl acetate; RPMI, RPMI 1640 medium. *P < 0.05 compared with ACh-exposed supernatant fluid.
Effects of ACh on LTB₄ and PAF production from bovine AMs. The measurement of LTB₄ by RIA revealed that AMs released LTB₄ in the baseline culture condition. The addition of ACh in a concentration of 100 µM

Fig. 4. Molecular-sieve column chromatography of released neutrophil (A), monocyte (B), and eosinophil (C) chemotactic activities from bovine AMs in response to 100 µM ACh. Nos. on top, molecular weight of indicated fraction.

Fig. 5. Effects of diethylcarbamazine (DEC), nordihydroguaiaretic acid (NDGA), AA-861, and cycloheximide (CYCLO) on release of neutrophil (A), monocyte (B), and eosinophil (C) chemotactic activities from bovine AMs (n = 8) in response to 100 µM ACh. *P < 0.05 compared with ACh-exposed supernatant fluid.
for 4 h caused a significant increase in LTB$_4$ release (P < 0.05; Fig. 7).

In response to ACh stimulation, PAF was detected in two samples out of six. However, the concentration of PAF was, at most, 40–50 pg/ml in two samples and <40 pg/ml in four samples out of six. Without stimulation, PAF was not detected in any samples (data not shown).

Table 2. Effects of IL-8 antibody on neutrophil chemotactic activity released from human AMs in response to ACh

<table>
<thead>
<tr>
<th>Group</th>
<th>Neutrophil Chemotactic Activity</th>
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<tr>
<td>Supernatant fluid from AMs from healthy volunteers</td>
<td>154 ± 12</td>
</tr>
<tr>
<td>Supernatant fluid from AMs from healthy volunteers + IL-8 antibody</td>
<td>147 ± 19</td>
</tr>
<tr>
<td>Supernatant fluid from ACh-stimulated AMs from healthy volunteers</td>
<td>287 ± 17</td>
</tr>
<tr>
<td>Supernatant fluid from ACh-stimulated AMs from healthy volunteers + IL-8 antibody</td>
<td>275 ± 24</td>
</tr>
<tr>
<td>Supernatant fluid from AMs from patients with interstitial lung disease</td>
<td>452 ± 141</td>
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<tr>
<td>Supernatant fluid from AMs from patients with interstitial lung disease + IL-8 antibody</td>
<td>225 ± 124</td>
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<tr>
<td>Supernatant fluid from ACh-stimulated AMs from patients with interstitial lung disease</td>
<td>624 ± 136</td>
</tr>
<tr>
<td>Supernatant fluid from ACh-stimulated AMs from patients with interstitial lung disease + IL-8 antibody</td>
<td>301 ± 128</td>
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Values are means ± SE in no. of cells/10 high-power fields. IL-8, interleukin-8; AM, alveolar macrophage.

Effects of ACh-receptor antagonists on the release of chemotactic activity from AMs in response to ACh. The involvement of the nicotinic receptor was examined with the nicotinic-receptor antagonist d-tubocurarine. The nicotinic-receptor antagonist did not inhibit the release of NCA, MCA, and ECA from AMs in response to ACh (data not shown). However, the muscarinic M$_3$-receptor antagonist 4-diphenylacetoxy-N-methylpiiperidine methiodide inhibited the release of NCA, MCA, and ECA (P < 0.05; Fig. 8). In contrast, the muscarinic M$_1$-receptor antagonist pirenzepine and the M$_2$-receptor antagonist gallamine did not inhibit the release of NCA, MCA, and ECA.
DISCUSSION

In the present study, AMs released NCA, MCA, and ECA in response to ACh in a dose- and time-dependent manner. NCA, MCA, and ECA were chemotactic by checkerboard analysis and were predominantly consistent with the low-molecular-weight lipoygenase-derived activity. LTB₄ was significantly released in response to ACh. PAF was released in a small amount. The ACh receptor responsible for the release of the chemotactic activity was the muscarinic M₃ receptor. These data suggest that ACh may play a role in bronchial inflammation by recruiting inflammatory cells into the bronchial lumen by stimulating AMs.

AMs release numerous chemotaxins, which include LTB₄ (3, 25, 37), PAF (2, 8), tumor necrosis factor-α (4), complement 5a (39), platelet-derived growth factor (27), and IL-8 (38). In the present study, the exact identification of the chemotactic activity released from AMs in response to ACh is not complete. However, nonspecific and 5-lipoxygenase inhibitors inhibited the release of chemotactic activity. An LTB₄-receptor antagonist inhibited chemotactic activity. The concentration of LTB₄ in the supernatant fluid, measured by RIA, was significantly increased. Thus LTB₄ was the predominant factor in NCA, MCA, and ECA released from AMs.

We performed neutrophil, monocyte, and eosinophil experiments in response to various concentrations of synthetic LTB₄. The neutrophil chemotactic responses were 96 ± 24 cells/10 HPF for 10⁻¹¹ M LTB₄, 187 ± 37 cells/10 HPF for 10⁻¹⁰ M LTB₄, and 272 ± 64 cells/10 HPF for 10⁻⁹ M LTB₄. Eosinophil and monocyte chemotaxis also showed similar data. The values were less than those for NCA, MCA, and ECA induced by the AM supernatant fluid. But there are multiple factors in the supernatant fluid as shown by partial characterization and molecular-sieve column. Other factors besides LTB₄ in the supernatant fluid may explain additional activity observed in the experiments.

Partial characterization of MCA and ECA indicated that the released activities were trypsin sensitive and inhibited by cycloheximide. MCA was also partially sensitive to heat. These data suggest that the chemotactic activities were, at least in part, composed of protein. The molecular-sieve column chromatography revealed that there were high-molecular-weight peaks of chemotactic activity in response to ACh. Although the identification of these chemotactic factors is uncertain, AMs released MCA and ECA composed of peptides in addition to LTB₄ in response to ACh. In contrast, the results of partial characterization, column chromatography, and lipoygenase inhibitors indicated that NCA was mainly composed of lipid. Because cycloheximide did not inhibit NCA, the release of lipid mediators might not need protein synthesis.

Because bovine ILs did not cross-react with human ILs and because it was difficult to obtain a sufficient number of human AMs, we used the supernatant fluids from human AM cultures from five healthy volunteers and five patients with interstitial lung disease. The IL-8 antibody blocked NCA of the supernatant fluid obtained from patients with interstitial lung disease in both unstimulated and ACh-stimulated AMs. However, in the supernatant fluid from healthy volunteers, the IL-8 antibody did not block ECA. The contribution of IL-8 as chemotactic activity might be minimal in the normal AMs in response to ACh. IL-3, IL-5, and GM-CSF antibodies did not block ECA or MCA of the supernatant fluids from all volunteers. Thus the involve-
ment of IL-3, IL-5, and GM-CSF was small. Sato et al. (33) have already reported that bradykinin stimulated AMs obtained from patients with interstitial lung diseases. Thus AMs from patients with interstitial lung disease may be so activated that they respond to various stimulations.

IL-8 is a cytokine with potent chemotactic activity for neutrophils and primed eosinophils. IL-8 was not produced from AMs in normal human volunteers in response to ACh. However, IL-8 was released from AMs in patients with active interstitial lung disease. ACh further augmented the release of IL-8 from AMs in these patients. These findings suggest that activated AMs from patients with bronchial asthma or other lung diseases may have the potential to release IL-8 in response to ACh.

PAF is a potent proinflammatory lipid mediator released by a number of cell types including epithelial cells, mast cells, endothelial cells (29), and macrophages. The involvement of PAF in the supernatant fluid as chemotactic activity was ~30% with the PAF-receptor antagonist. However, PAF was only slightly detected in the AM supernatant fluid in the present study. The concentration of PAF in the supernatant fluid was less than that of the chemotactic activity in the present study. We speculate that the effects of the PAF-receptor antagonist may be due to the inhibition of action of PAF as a potentiator by way of a neutrophil (inflammatory cell) surface receptor rather than the direct inhibitory effect of PAF as chemotactant in the supernatant fluid.

Wessler et al. (40) reported that dissected human bronchi obtained by thoracotomy release ACh by electrical stimulation to the salt solution. This shows that ACh diffuses to the alveolar spaces and bronchial lumens. They also measured the tissue content of ACh. The concentration of ACh at the neuromuscular junction was reported to be 10^-4 to 10^-3 M. In the present experiment, the lowest dose of ACh to stimulate AMs was 1.0 µM. Although the exact concentration of ACh in BALF or lung tissue is uncertain, the concentration of ACh seems to be high enough to stimulate AMs.

Anticholinergic drugs often dilate airways in patients who have asthma and COPD. Recent studies suggest that cholinergic tone is increased in COPD and asthma (11, 24, 41). Thus the rationales of the anticholinergic agent for asthma and COPD are due to the inhibition of bronchomotor tone. However, it was reported (21) that ACh stimulated the release of NCA and MCA from bovine bronchial epithelial cells. Sarali and Chan-Yeung (31) reported that ACh stimulated the release of 15-hydroxyeicosatetraenoic acid from human airway epithelial cells. Because ACh stimulates AMs and bronchial epithelial cells to release inflammatory cell chemotactic activity and because inflammatory cells play an important role in bronchial hyperreactivity, the present results suggest that anticholinergic drugs may additionally inhibit bronchial hyperreactivity by preventing airway inflammation.

In conclusion, ACh stimulated AMs to release NCA, MCA, and ECA. The released activities were mainly consistent with the low-molecular-weight lipoxygenase-derived activity. The receptor responsible for the release of chemotactic activities in response to ACh was the M3 receptor. These results suggest that ACh may play a role in inflammatory cell recruitment into the airways by stimulating AMs to release chemotactic activity.

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