Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity

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Koyama, Sekiya, Etsuro Sato, Hiroshi Nomura, Keishi Kubo, Sonoko Nagai, and Takateru Izumi. Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. J. Appl. Physiol. 86(5): 1528–1534, 1998.—We investigated a role of neuroregulation in the release of eosinophil chemotactic activity (ECA) from bovine bronchial epithelial cells (BBEC). BBEC were stimulated with acetylcholine (ACh) and substance P (SP), and the supernatant fluids were tested for ECA by a blind-well chemotactic chamber technique. BBEC released ECA in response to ACh and SP in a dose- and time-dependent manner. Checkboard analysis showed that ECA in regard to ACh and SP was chemotactic rather than chemokinetic. Partial characterization revealed that ECA involved both lipids and peptides. The release of ECA in response to ACh and SP was inhibited by nonspecific and 5-specific lipoygenase inhibitors and by cycloheximide (P < 0.01). Molecular-sieve column chromatography revealed that these mediators induced three molecular mass peaks (near 25 kDa, 9 kDa, and 400 Da, respectively). The lowest peak, which represented the predominant activity, was blocked by leukotriene B4 receptor antagonist (P < 0.01) but not by platelet-activating factor receptor antagonist. The release of leukotriene B4 in the supernatant fluids was increased in response to ACh and SP stimulation (P < 0.01). Platelet-activating factor was not detected. These results raise the possibility of a role of neuroregulation for the elaboration of ECA in the airway.

bronchial epithelial cell; eosinophil chemotaxis; leukotriene B4

AIRWAY EOSINOPHILIC INFLAMMATION is one of the most specific changes in the pathology of asthma (10) and a major marker of the inflammatory activity in the disease (31). An increased number of eosinophils is usually found in the sputum (13), in the bronchoalveolar lavage fluid (BALF) (8, 37), in the airway epithelia and submucosa (5, 31), and, frequently, in the blood (21) of asthmatic patients. Although the lymphocytes and the eosinophils are the predominant infiltrating cells in airway mucosa of asthmatic subjects (10), the eosinophil is believed to be a primary cell responsible for the development of many features of asthma, including damage and desquamation of the respiratory epithelia (7), airway hyperresponsiveness (37), and allergen-induced late asthmatic reaction (8). Furthermore, the degree of infiltration of the bronchial wall by eosinophils is related to the clinical severity of asthma (6).

The extent of eosinophil accumulation at a specific site in the organism is regulated by several mechanisms. One requirement is the availability of a sufficient number of cells in the blood pool. Although the mechanisms that govern the egress of eosinophils from the bone marrow are not fully understood, T-lymphocyte-dependent and -independent mechanisms have been proposed (4). The second major requirement is the production of some factors at the specific site, which signal the eosinophil to leave the bloodstream. The administration of antibody to one of the adhesion molecules, intercellular adhesion molecule-1, inhibited the eosinophil accumulation and the development of hyperreactivity, suggesting a role of the eosinophil-endothelial cell interaction (38). However, it is assumed that these signals involve specific eosinophil chemotactic factors (16).

Because the actions of substance P (SP) include vasodilation, vascular leakage, airway secretion, and contraction of smooth muscles (2, 3) and because acetylcholine (ACh) regulates airway tone and secretion, SP and ACh are thought to be potent factors in asthma (3). Bovine bronchial epithelial cells (BBEC) participate in the inflammatory response in the airway by releasing chemotactic activity for neutrophils and monocytes (26–29, 36). However, the role of neuroregulation of the airway cells for the elaboration of eosinophils is unknown. In the present study, we demonstrated that BBEC released eosinophil chemotactic activity (ECA) in response to ACh and SP. These results may suggest the possibility of a role of neuroregulation for the elaboration of ECA, in addition to the known neural involvement in airway pathophysiology of asthma.

METHODS

Culture and identification of BBEC. BBEC were isolated and cultured by a modification of the methods of Wu and colleagues (39). Briefly, bovine lungs were obtained from freshly killed cows. The alveolar and interstitial structures were removed by teasing, and the bronchi were sectioned into 4- to 7-cm-long pieces. The sectioned bronchi were then incubated overnight at 4°C in Eagle's minimum essential medium (GIBCO, Grand Island, NY) with 0.1% bacterial protease (Streptomyces griseus, type XIV; Sigma Chemical, St. Louis, MO). The BBEC were recovered by washing the epithelial surface of the bronchi. The recovered cells were suspended at 2.0 × 10⁶ cells/ml in medium 199 (GIBCO) supplemented as previously described (28). Then 1.5 ml of BBEC suspension were added to a 35-mm-diameter tissue-culture dish (Corning, Corning, NY) and cultured at 37°C in 5% CO₂ atmosphere. The tissue-culture dish was not coated with any types of substratum of extracellular matrix protein. With the use of these techniques, the cultured cells were identified as epithelial cells by staining with anti-keratin antibody (ICN Immunological, Lisle, IL), and these cells were histologically and functionally competent as previously re-
ported (28, 29). BBEC monolayers cultured as above formed dome by phase-contrast microscope, and tight junctions were assessed by electron microscope and blocked albumin permeability. Transepithelial resistance was 412–487 Ω·cm² (457 ± 32 Ω·cm², n = 6 experiments) cultured on Transwell plates.

Exposure of BBEC to ACh and SP. Medium was removed from cells by washing them twice with serum-free medium, and BBEC were incubated with medium supplemented without fetal calf serum (FCS; GIBCO), both in the presence and absence of ACh and SP (0, 0.1, 1.0, 10, 100, and 1,000 µM, respectively) and cultured at 37°C in a humidified 5% CO₂ atmosphere for 4, 6, 12, 24, 48, and 72 h. These agents did not cause BBEC injury (no deformity of cell shape, no detachment from tissue-culture dish, and >95% of cells viable by trypan blue exclusion) after 72-h incubation at the maximal doses.

Because BBEC made domes, formed tight junctions, and polarized (26, 27), we cultured BBEC on Transwell plates, which had 24 wells and were separated by polycarbonate filters that had a diameter of 6.5 mm and a poresize of 0.3 µm. After cells reached confluence and BBEC monolayers had negligible BSA permeability compared with filter alone, BBEC were stimulated from the basolateral side with 100 µM of ACh and SP for 48 h. The upper and lower chambers were filled with 500 µl of serum-free medium to avoid pressure gradient. The supernatant fluids in the upper and lower chambers were harvested after 48 h for chemotaxis assay.

The culture supernatant fluids were harvested and frozen at −80°C until assayed. At least five separate BBEC supernatant fluids were harvested from cultures obtained from different animals for each experimental condition.

Measurement of ECA. Eosinophil isolation was performed by a minor modification of the method by Hansel and co-workers (18), who used a magnetic cell-separation system. The purity of eosinophils as counted by Randolph’s stain was >94%, and the viability was >98%. Isolated eosinophils were washed twice with PIPES buffer containing 1% FCS. The eosinophils were suspended in Gey’s balanced salt solution containing 2% BSA at pH 7.2 to give a final concentration of 3.0 × 10⁶ cells/ml. These suspensions were used in the chemotaxis assay.

The chemotaxis assay was performed in 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD), as has been described (19). Each sample was tested in duplicate. A polycarbonate filter (Nucleopore, Pleasanton, CA) with a pore size of 5 µm was placed, and the chamber was incubated in humidified 5% CO₂ at 37°C for 120 min. After the incubation, the chamber was disassembled, and the filter was fixed, stained with Diff-Quik (American Scientific Products, McGraw Park, IL), and mounted on a glass slide. Cells that completely migrated through the filter were counted in five random high-power fields (HPF: ×1,000) from each duplicate well. Chemotactic response was defined as the mean number of migrated cells per HPF. Medium 199 without FCS was incubated identically with BBEC, and the supernatant fluids harvested were used to determine background eosinophil migration. Leukotriene B₄ (LTB₄; at 10⁻⁷ M in supplemented medium 199; Sigma Chemical) and platelet-activating factor (PAF; at 10⁻⁷ M in supplemented medium 199) were used as positive controls for eosinophils.

To determine whether the migration was due to a movement along a concentration gradient (chemotaxis) or to a stimulation to randomly migrate (chemokinesis), a checkerboard analysis (40) was performed with BBEC supernatant fluid stimulated by using 100 µM of ACh and SP for 72 h. To do this, various concentrations of BBEC supernatant fluids (1:27, 1:9, 1:3, 1:1) were placed above and below the membrane.

Partial characterization of the released ECA. Because ECA was detected in BBEC culture supernatant fluids, partial characterization was performed by utilizing supernatant fluids harvested at 72 h in response to ACh and SP. Sensitivity to protease was tested by incubating BBEC culture supernatant fluids with trypsin (final concentration 100 µg/ml; Sigma Chemical) 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 before the chemotactic assessment. The lipid solubility was evaluated by mixing BBEC culture supernatant fluids twice with ethyl acetate, decanting the lipid phase after each extraction, evaporating the ethyl acetate to dryness, and resuspending the extracted material in the supplemented medium 199. Heat sensitivity was determined by maintaining a temperature of 98°C for 15 min.

Effects of metabolic determinants on ECA release. BBEC are capable of releasing arachidonic acid metabolites that may account for the released ECA. Therefore, BBEC were pretreated by nonspecific and 5-specific lipoxigenase inhibitors: nordihydroguaiaretic acid (NDGA; 100 µM, Sigma

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![Fig. 1. Dose-dependent release of eosinophil chemotactic activity in response to acetylcholine (ACh; A) and substance P (SP; B) from bovine bronchial epithelial cell monolayer (n = 6 experiments). Values are means ± SE. *P < 0.05 compared with supernatant fluids without stimuli.](http://jap.physiology.org/Downloaded/from/10.220.33.5)
Measurement of LTB4 was performed by radioimmunoassay, LTB4-receptor antagonist (ONO-4057, Ono Pharmaceutical, Tokyo, J apan) for 30 min before the addition of stimuli, and their effects on the release of ECA were examined. To evaluate the role of protein synthesis in the release of ECA, BBEC were pretreated with cycloheximide (10 µg/ml, Sigma Chemical). At these concentrations, NDGA, DEC, and AA-861 inhibited the release of lipoygenase metabolites from BBEC in response to lipopolysaccharide (26, 27). These inhibitors did not cause cytotoxicity to BBEC after 72-h incubation.

Partial purification of ECA by column chromatography. To determine the approximate molecular mass of the chemotactic activity released from BBEC in response to ACh and SP, molecular-sieve column chromatography was performed by using Sephadex G-75 (75 × 1.25 cm, Pharmacia, Piscataway, NJ). At a flow rate of 6 ml/h, the BBEC culture supernatant fluid was eluted with PBS, and every other fraction after the void volume was evaluated for ECA in duplicate.

To evaluate the responsible chemotactic activity in the lowest molecular mass peak separated by column chromatography, LTB4-receptor antagonist (ONO-4057, Ono Pharmaceutical, Tokyo, Japan) and PAF-receptor antagonist (TCV-309, Takeda Pharmaceutical, Tokyo, Japan) were used at the concentration of 10−5 (25, 35).

Measurement of LTB4 and PAF in the supernatant fluid. The measurement of LTB4 was performed by radiolmmunoassay (RIA), according to manufacturer’s directions (1), by using anti-LTB4 serum [5, 6, 8, 9, 11, 12, 14, 15-3H(N)]LTB4 and synthetic LTB4, which were purchased from Amersham (Arlington Heights, IL). Briefly, ethanol samples were centrifuged at 5,500 g at 0°C. Then, the supernatant fluids were evaporated under N2 gas at 37°C. To each sample, 10 ml distilled water were added. These samples were acidified to pH 4.0 with 0.1 M HCl and applied to Sep-Pak C18 columns (Waters Associates, Milford, MA). The columns were washed twice with a mixture of 10 ml distilled water and 20 ml petroleum ether, then eluted with 15 ml ethanol. These eluates were dried with N2 gas at 37°C, then redissolved in a combination of 20 µl methanol and 180 µl RIA buffer [50 mM Tris-HCl buffer containing 0.1% (wt/vol) gelatin, pH 8.6]. [3H]LTB4 was diluted in RIA buffer, and 100-µl aliquots containing −4,000 dpm were mixed with 100 µl of standards or samples in disposable siliconized tubes. Anti-LTB4 serum diluted in 100 µl of RIA buffer were added to give a total incubation volume of 0.4 ml. The mixture was incubated at 4°C for 18 h. Free LTB4 was adsorbed onto dextran-coated charcoal. The supernatant, containing the antibody-bound LTB4, was decanted into scintillation counter after centrifugation for 15 min at 2,000 g. Scintillation fluid (AquaZol 2; DuPont NEN, Boston, MA) was added, and radioactivity was counted by scintillation counter (TriCarb-3255, Tackard, IL) for 4 min.

Table 1. Checkerboard analysis of released eosinophil chemotactic activity in response to acetylcholine

<table>
<thead>
<tr>
<th>Lower Well</th>
<th>M-199</th>
<th>1:27</th>
<th>1:9</th>
<th>1:3</th>
<th>1:1</th>
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<td>M-199</td>
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<td>15.3±2.4</td>
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<td>14.8±3.3</td>
<td>12.1±3.1</td>
<td>16.83±2.5</td>
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<td>23.5±2.5</td>
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Table 2. Checkerboard analysis of released eosinophil chemotactic activity in response to substance P

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Values are means ± SD of cells/high-power field. Checkerboard analysis of a bronchial epithelial cell culture supernatant fluid harvested after 72 h in response to acetylcholine at concentration of 100 µM. Vertical column represents dilutions of bronchial epithelial cell supernatant fluid placed in lower wells; horizontal row represents dilutions of supernatant fluid in upper wells. Medium 199 (M-199) represents supplemented culture media without fetal calf serum.
standard similar to the methods of measurement of LTB₄ (30).

Statistics. In experiments that were multiphased, significa-
cant differences between groups were tested by using one-
way analysis of variance. Duncan’s multiple-range test was
applied to data at specific time and dose points. In experi-
ments in which a single measurement was made, the differ-
ence between groups was tested for significance by using
Student’s paired t-test. In all cases, a P value <0.05 was
considered significant. Data in Figs. 1–7 and Tables 1 and 2
are expressed as means ± SE.

RESULTS

Release of ECA from BBEC. BBEC released ECA in
response to ACh and SP in a dose-dependent fashion
(Fig. 1, A and B). The lowest dose that stimulated
BBEC in response to ACh and SP was 0.1 µM. An

Fig. 3. Partial characterization of released eosinophil chemotactic activity in response to ACh (A) and to SP (B) from bovine bronchial epithelial cell monolayer (n = 6 experiments). EA extracted eosinophil chemotactic activity that was extracted into ethyl acetate, dried, and resuspended in M-199 medium. EA extractant, remaining eosinophil chemotactic activity in supernatant fluids after ethyl acetate extraction. Values are means ± SE. *P < 0.01 compared with crude sample.

Fig. 4. Effects of nordihydroguaiaretic acid (NDGA), diethylcarba-
azine (DEC), AA-861, and cycloheximide on release of eosinophil
chemotactic activity in response to ACh (A) and to SP (B) from bovine
bronchial epithelial cell monolayer (n = 6 experiments). Values are
means ± SE. *P < 0.01 compared with stimulus alone.

Fig. 5. Molecular-sieve column chromatographic findings of released
eosinophil chemotactic activity in response to ACh (A) and SP (B)
from bovine bronchial epithelial cell monolayer. Data are a represen-
tative profile of 4 experiments.
increase in concentrations of ACh and SP progressively augmented the release of ECA, culminating at 100 µM. The release of ECA began 12 h after exposure to ACh and SP (Fig. 2, A and B), and the released activity reached a plateau at 48 h (Fig. 2, A and B). The released ECA harvested from BBEC on Transwell plates from both chambers did not show significant difference [upper chamber 22.5 ± 3.2 (ACh), 20.7 ± 4.2 (SP) vs. lower chamber 18.4 ± 4.9 (ACh), 22.8 ± 4.6 (SP) cells/HPF, n = 6, P > 0.05].

The chemotactic activities in response to LTB 4 and PAF were 58.4 ± 8.7 and 68.3 ± 3.2 eosinophils/HPF, respectively. ACh and SP by themselves did not show any chemotactic activity for eosinophils in the culture medium without BBEC and when incubated identically (data not shown).

Checkerboard analysis revealed that BBEC supernatant fluids stimulated by ACh and SP induced eosinophil migration in the presence of concentration gradient across the membrane and a smaller increase in the absence of gradient (Tables 1 and 2). Thus the migration in response to ACh- and SP-stimulated BBEC supernatant fluids was predominantly chemotactic, rather than chemokinetic.

Partial characterization of the released ECA. The released chemotactic activity obtained from BBEC supernatant fluids incubated with 100 µM of ACh and SP for 72 h was partially sensitive to heat and lipid extraction (Fig. 3, A and B). Trypsin digestion and ethyl acetate extraction enhanced the ECA as compared with the crude sample.

Effects of metabolic inhibitors on the release of ECA. BBEC incubated with 100 µM of ACh and SP in the presence of the lipoxygenase inhibitors NDGA, DEC, and AA-861 showed a significant decrease in the release of ECA (Fig. 4, A and B) into the supernatants. NDGA, DEC, and AA-861 did not have any effects on LTB4- and PAF-induced eosinophil chemotaxis (data not shown). Cycloheximide partially inhibited the release of ECA in response to ACh and SP (Fig. 4, A and B).

Partial purification of ECA. Molecular-sieve column chromatography with the use of Sephadex G-75 revealed that the released ECA was heterogeneous in size (Fig. 5, A and B). At least three peaks of activity were identified by column chromatography, with the estimated molecular mass after BSA (66.2 kDa), after cytochrome-c (12.3 kDa), and an additional peak that eluted near quinacrine (450 Da), respectively. The lowest molecular mass peak near quinacrine represented the majority of the activity.

Effects of LTB4 and PAF-receptor antagonists on the released ECA. The activity of the lowest molecular mass ECA separated by G-75 column chromatography was partially inhibited (>50%) by the addition of LTB4-receptor antagonist ONO-4057 (Fig. 6, A and B). The PAF-receptor antagonist, TCV-309, did not inhibit this lowest molecular mass ECA. These receptor antagonists at the concentration of 10−5 M completely inhibited the eosinophil migration in response to LTB4 and PAF at a concentration of 10−7 M (15.6 ± 2.4 and 13.4 ± 3.1 eosinophils/HPF, respectively) but showed no inhibitory effects on eosinophil chemotaxis induced by activated serum (data not shown).
Concentrations of LTB₄ and PAF in the supernatant fluids. The concentrations of LTB₄ in the supernatant fluids in response to ACh and SP at the concentrations of 100 µM for 24-h incubation were significantly increased compared with control (Fig. 7, P < 0.01). In contrast, PAF in BBEC supernatant fluids in control and in response to ACh and SP was not detected.

**DISCUSSION**

Many eosinophil chemotactic factors are released from a variety of cells. Recently, cytokines, including granulocyte-macrophage colony-stimulating factor; Regulated on Activation Normal T cells, Expressed and Secreted; and interleukin (IL)-3, IL-4, IL-5, and IL-8, have been reported to play roles in eosinophilopoiesis and differentiation (11, 32). LTB₄, a potent inflammatory cell chemotactic factor, can be released from a variety of cells, including neutrophils, eosinophils, alveolar macrophages (34), and mononuclear cells, and is present in the BALF recovered from asthmatic patients (1). PAF, platelet factor 4, activated complement, and fibrin-degradation product have also been suggested to play a role in eosinophil recruitment observed in asthma (9, 12, 23). Two tetrapeptides and histamine have also been suggested as eosinophil chemotactic factors (14). However, the participation of the airway cell in the recruitment of eosinophils and the role of neuroregulation for the ECA release are unknown. In the present study, BBEC released ECA in response to ACh and SP in a dose- and time-dependent fashion. The released activity was chemotactic, as certified by checkerboard analysis. Partial characterization revealed that the activity was predominantly lipid extractable. The column chromatography showed that the lowest molecular mass chemotactic peak was that of predominant activity. Both the 5-lipoxygenase inhibitor and LTB₄-receptor antagonist blocked ECA. Finally, the release of LTB₄ was significantly increased in response to ACh and SP, reaching a level that induced eosinophil chemotaxis. These data suggest that neuroregulation of ECA release from the airway cells may play a role in eosinophil recruitment into the airway.

SP and ACh are thought to be a potential neuroregulator in bronchial asthma (3). These substances have direct effects on airway functions, including the increases in airway hyperreactivity, airway smooth muscle cell contraction, and airway secretion (3, 4). On the other hand, several lines of evidence support the concept that eosinophil infiltration may lead to an increase in airway hyperreactivity. In patients with asthma, the disease severity correlates with the degree of bronchial wall infiltration of eosinophils (6). The study of BALF demonstrates a correlation between the presence and activity of eosinophils and the severity of the asthmatic disease (6). Patients who developed a late asthmatic reaction have a raised number of blood eosinophils and a raised eosinophil number in BALF (8). Thus the release of ECA from BBEC in response to ACh and SP suggests a role for the airway cells in the increase of eosinophil infiltration in the bronchial wall and lumen in patients with asthma and may suggest the possibility of neuroregulation of the eosinophil recruitment, in addition to the known neural regulation of the airway functions.

The released chemotactic activity from BBEC in the present study is not yet completely characterized. However, the predominant ECA was lipid extractable. The nonspecific and 5-specific lipoxygenase inhibitors blocked the release of chemotactic activity, suggesting that the activity may be derived from the 5-lipoxygenase pathway. The lowest molecular mass peak ECA was blocked by LTB₄-receptor antagonist. Finally, immunoreactive LTB₄ in the supernatant fluids increased to a level that was chemotactic for eosinophils. On the basis of these lines of evidence, LTB₄ is the predominant ECA. In support of this concept is the fact that LTB₄ and 12-lipoxygenase-pathway products can be released from bovine airway cells (17, 26, 27), and LTB₄ and 15-dihydroxyeicosatetraenoic acid are released from dog (24) and human tracheal epithelial cells (22), respectively. These lipoxygenase metabolites are chemotactic for eosinophils (1, 15, 24, 33). The release of lipoxygenase metabolites is species specific and differs in response to a variety of stimuli, even in the same species. However, it has many common biological characteristics. Thus neuroregulation of the airway cells may play a role in the release of lipoxigenase-derived ECA.

The ethyl acetate extraction and trypsin digestion of the supernatant fluids resulted in the increase in chemotactic activity, compared with the crude sample. Although the mechanisms underlying this potentiation of ECA are unknown, the presence of eosinophil-migration inhibitor in the supernatant fluids may be one of the possible explanations. The removal by ethyl acetate extraction or destruction by trypsin of eosinophil-migration inhibitor resulted in the potentiation of ECA. If this is the case, this inhibitory factor(s) may prolong eosinophil retention in the bronchial lumens.

In conclusion, BBEC released ECA in response to ACh and SP in a dose- and time-dependent fashion. The released activity was chemotactic, as shown by checkerboard analysis and low-molecular-mass lipoxygenase-derived activity, i.e., LTB₄. The results may suggest the possibility of a role for neuroregulation of the eosinophil recruitment in the bronchial wall and lumen, in addition to the known neural regulation of the airway functions.

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