

Adhesive Explant Culture of Allergic Nasal Mucosa: Effect of Emedastine Difumarate, an Anti-allergic Drug

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ABSTRACT—Allergic reaction of the nose comprises of an immediate and a late reaction. To evaluate nasal allergic reactions, many experiments have been performed by investigators. In this study, we performed a new tissue culture technique (adhesive explant culture) to analyze the migration of cells into the culture medium from the cultured allergic nasal mucosa in response to an allergen. Basophilic cells (mast cells and basophils) and eosinophils, which were released into the culture medium after the allergen challenge, were evaluated by the analysis of histamine and eosinophil cationic protein (ECP) content in the culture medium. Histamine and basophilic cells in the culture medium were more abundant in the immediate phase (within 30 min) after challenge than in the late phase (from 30 min to 10 hr). On the other hand, ECP and eosinophils in the culture medium were more abundant in the late phase than in the immediate phase. The increase of histamine content in both phases were not inhibited by pre-treatment of emedastine difumarate (EME), an anti-allergic drug. However, the increase of ECP in the late phase was inhibited by pre-treatment with EME. Moreover, the number of EG2-positive cells was also decreased by pre-treatment with EME. These results suggest that EME might lower the activation of eosinophils in the late phase of the allergic reaction. The present study also indicates that this adhesive explant culture system is useful model for studying the cellular allergic responses to drugs *ex vivo*.

Keywords: Nasal allergy, Explant culture, Eosinophil, Basophilic cell, Anti-allergic drug

Allergic reactions of the nasal mucosa comprises of an immediate and a late reaction (1). The nasal allergic reaction is complicated and includes submucosal glandular secretion, vasodilatation primarily involving the venous system, increased vascular permeability, and autonomic nervous reflexes. Each response is induced by chemical mediators released from inflammatory cells (2). Immediate allergic reactions are primarily induced by histamine released from mast cells, while the late phase of the reaction involves leukotrienes released from eosinophils (3) and cytokines released from lymphocytes (4). To investigate the pathophysiology of nasal allergy, we devised a modified explant culture (adhesive explant culture) *ex vivo* for monitoring the migrating cells in the immediate (within 30 min) and the late allergic responses (from 30 min to 10 hr) of the nasal mucosa following allergen (Ag) challenge. Many investigators have analyzed the immediate and late phases of the allergic response using *in vivo* systems. Trials of new anti-allergic drugs, however, are difficult using *in vivo* systems. Thus we devised a modified explant culture system of the human nasal mucosa. The

objectives of the present study were to assess the effects of emedastine difumarate on the migration and activation of basophilic cells (mast cells and basophils) and eosinophils in the allergic nasal mucosa using the adhesive explant culture system and to determine the usefulness of this *ex vivo* system for developing agents effective against nasal allergy.

MATERIALS AND METHODS

Materials

Specimens of the nasal mucosa were obtained from the inferior turbinate of 4 patients with non-seasonal nasal allergy to house dust mite diagnosed on the basis of medical history rhinoscopy and allergy tests (eosinophil count in nasal discharge and blood, total serum IgE, serum antigen-specific IgE and nasal mucosal provocation test). All 4 patients exhibited a positive late phase reaction after *in vivo* Ag challenge. All of them complained of nasal obstruction and gave informed consent to nasal surgery and to the use of resected nasal mucosa for this study.

Culture and Ag challenge

Nasal mucosa was excised under local anesthesia achieved by injection of 1% lidocaine with 500,000-fold diluted epinephrine into the nasal mucosa, and not by superficial anesthesia with gauze application. The resected nasal mucosa was cut into 4 pieces (about 2×1 cm with mucosal surface, about 180 mg average wet weight), and these pieces were washed with saline for several seconds and then pre-incubated for 1 hr with RPMI medium (control) or RPMI medium containing 10^{-5} – 10^{-7} M emedastine difumarate (EME; Kanebo Pharmaceutical Co., Ltd., Tokyo). The mucosal specimen was then immediately fixed in a position sectioned side down onto a Petri dish with fibrin glue. Fibrin glue makes a clot at the cut surface of the nasal mucosa. Subsequently, 5 ml of RPMI medium containing 0.1 mg/ml (12 μ g PN/ml, final concentration) of lyophilized house dust mite Ag (Torii Pharmaceutical Co., Ltd., Tokyo) was added to each dish to induce allergic responses. Thirty minutes later, the supernatant was collected and stored for assessment of the immediate responses. Then each tissue sample was incubated in 5 ml of RPMI medium for an additional 9.5 hr in a moist chamber for assessment of the late phase responses (Fig. 1). The supernatant was collected and subjected to 3 cycles of freezing and thawing to cause complete release of chemical mediators from the inflammatory cells as previously reported (5).

Quantitative determination of histamine and eosinophil cationic protein (ECP)

To estimate the activity of migrating eosinophils and basophilic cells (mast cells and basophils), the culture supernatants obtained at 30 min and at 10 hr were assayed to determine the ECP and histamine content, respectively, using commercial RIA kits (Pharmacia, Piscataway,

NJ, USA). Individual results were expressed as the total histamine (released histamine and basophilic cells) and ECP (released ECP and eosinophils) from the individual specimens and the percentages relative to spontaneous release from that observed during pre-incubation (natural exposure). Inhibitory effect of EME was expressed as percentages relative to mediator release and/or migration of mediator-containing cells by control Ag challenge for each time point.

Immunohistochemistry

After investigating the allergic response to Ag challenge at 10 hr, the nasal mucosa was washed with saline and frozen sections were prepared for immunohistochemical staining with the anti-human EG2 mAb (Pharmacia) by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Frozen sections were fixed with acetone and reacted at 4°C overnight with anti-human EG2 mAb as the primary antibody. After washing, the sections were reacted for 30 min with the rabbit anti-mouse IgG antibody as the secondary antibody. Subsequently, the sections were incubated in the APAAP reagent. Each section was finally incubated with the enzyme substrate and was counter-stained with hematoxylin. The positive cells were counted in 20 high performance fields (HPF) at a magnification of 200 $\times$. The mean EG2-positive cell/HPF and standard deviation were calculated.

Statistical analyses

The results of histamine content and ECP content in culture medium are expressed as the mol/tissue (histamine), the μ g/tissue (ECP), and the % content compared to spontaneous release (natural exposure). The effect of EME expressed as the % release of mediators and/or mediator-containing cells was compared to that at the same time points of the control (cont). All data are expressed as the mean $\pm$ S.D. Statistical analysis was performed by the Student's *t*-test compared to the control.

RESULTS

Migrating cell analysis in this adhesive explant culture system *ex vivo*

The amount of histamine in the culture medium (basophilic cells and/or released histamine) within 30 min was 0.97 ± 0.57 nmol/tissue and $68.5 \pm 53.6\%$ of that released spontaneously (natural exposure) from the individual mucosal sections. From 30 min to 10 hr, the histamine content was 3.10 ± 1.01 nmol/tissue and $251.2 \pm 90.4\%$ of the spontaneous level (Fig. 2A). This indicates that a greater amount of basophilic cells and/or histamine itself was released during the late phase than during the immediate phase, but the collecting time was different

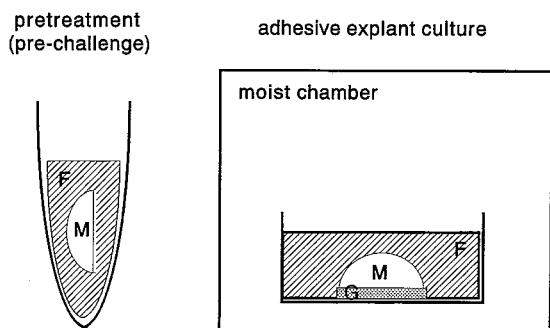


Fig. 1. Illustration of adhesive explant culture. Left: After washing resected nasal turbinate with saline, the nasal mucosa (M) was pre-incubated for 1 hr in RPMI medium with or without emedastine difumarate (EME: F). Right: Nasal mucosa (M) specimens were fixed in a position sectioned side down onto a Petri dish with fibrin glue (G). Subsequently RPMI containing lyophilized house dust mite antigen (F) was added.

(30 min vs 9.5 hr). Eosinophils and/or released ECP in culture medium within 30 min was only $0.011 \pm 0.002 \mu\text{g}/\text{tissue}$ and $15.6 \pm 9.9\%$ of spontaneous release, but eosinophils and/or released ECP ($0.087 \pm 0.034 \mu\text{g}/\text{ml}$, $271.5 \pm 284.7\%$) increased from 30 min to 10 hr (Fig. 2B).

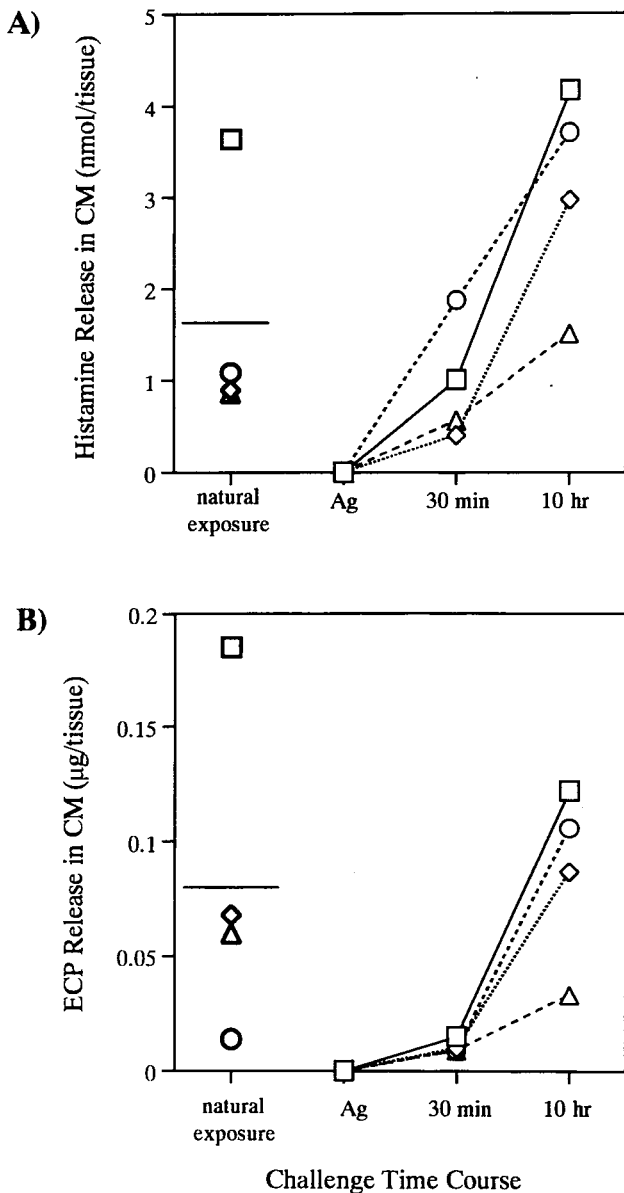


Fig. 2. Mediators in culture medium (CM) after allergen (Ag) challenge ex vivo. A: Released histamine and basophilic cells in CM. Time course (0, 30 min, 10 hr) of histamine release (nmol/tissue) after Ag challenge in patient (Pt.) No. 1 (□), Pt. No. 2 (◇), Pt. No. 3 (○) and Pt. No. 4 (△). Spontaneous release of histamine and/or basophilic cells (bar: mean, 1.63 ± 1.17 nmol/tissue) is shown by a bold symbol (natural exposure). B: Released ECP and eosinophils in CM. Time course (0, 30 min, 10 hr) of ECP release ($\mu\text{g}/\text{tissue}$) after Ag challenge in each case presented same as panel A. Spontaneous release of ECP and/or eosinophils (bar: mean, $0.082 \pm 0.063 \mu\text{g}/\text{tissue}$) is shown by a bold symbol (natural exposure).

The effect of EME for migrating cells in the same culture system

The results of histamine content suggested that pretreatment with EME did not suppress the release of histamine-containing cells and/or histamine itself from the nasal mucosa during either phase (Fig. 3: A and B). The control of histamine content at each time point was 0.97 ± 0.57 and 3.10 ± 1.01 nmol/tissue, respectively, for

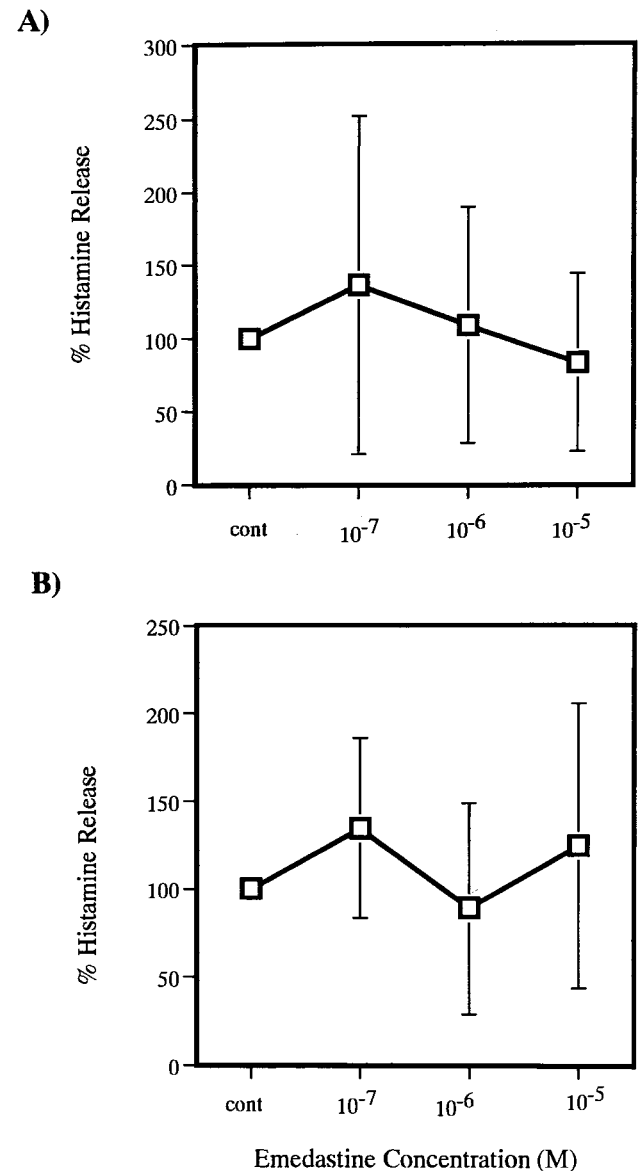


Fig. 3. Inhibition of histamine in culture medium after Ag challenge ex vivo by EME. A: Inhibition of histamine release and basophilic cells to culture medium in immediate phase (within 30 min) by emedastine difumarate (EME). Y axis represent the value of % release. Bars represent S.D. B: Inhibition of histamine release and basophilic cells to culture medium in late phase (from 30 min to 10 hr) by EME. Y axis represents the value of % release. Bars represent S.D.

the immediate and late phases. EME did not alter released ECP and/or eosinophil release during the immediate phase (data not shown), but suppressed those release during the late phase in a concentration-dependent manner, significantly (EME; 10^{-5} M, $P < 0.05$) (Fig. 4). The control of ECP content at 10 hr was 0.087 ± 0.034 μ g/ml.

Immunohistochemistry for EG2 and the effect of EME

Immunohistochemical staining showed that the majority of EG2-positive cells in the nasal mucosa at 10 hr after Ag challenge were present immediately beneath the epithelium in this patient (Pt.) No. 4 (Fig. 5A). The results of all cases indicated a similar distribution. EME at 10^{-6} and 10^{-7} M slightly inhibited the cell migration and EG2-positive cells, EME at 10^{-5} M did not (Fig. 5: B–D). The distribution of EG2-positive cells with EME treatment, however, was almost the same as that of the control (Fig. 5: B and C). The number of EG2-positive cells in the nasal mucosa as counted in 20 high performance fields showed a tendency to be decreased by EME pre-treatment in a dose-dependent manner in all cases (Fig. 6). EME at 10^{-5} M significantly suppressed the increase in the number of activated eosinophils in Pt. No. 1 and 3 ($P < 0.05$) (Fig. 6).

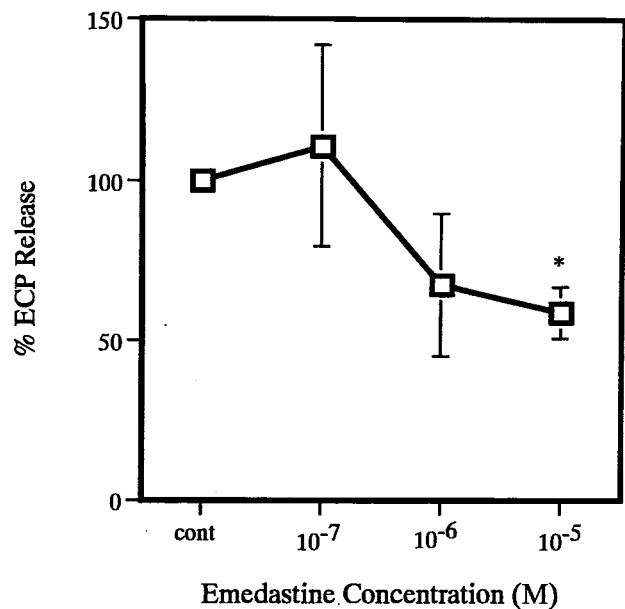


Fig. 4. Inhibition of ECP in culture medium after Ag challenge ex vivo by EME. Inhibition of released ECP and eosinophils to culture medium in late phase (from 30 min to 10 hr) by EME. Y axis represent the value of % release. Bars represent S.D. * $P < 0.05$, compared with the control (cont).

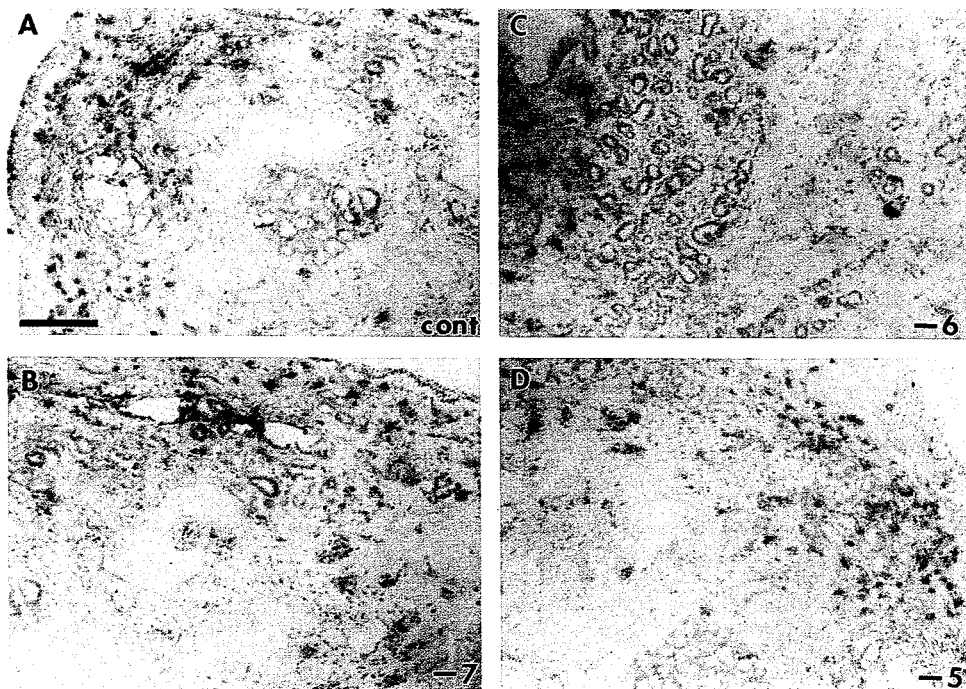


Fig. 5. Immunohistochemistry of EG2-positive cells in nasal mucosa at 10 hr after in vitro Ag challenge in Patient No. 4. A: Nasal mucosa pre-incubated by RPMI only. B: Nasal mucosa pre-incubated by RPMI with 10^{-7} M EME. C: Nasal mucosa pre-incubated by RPMI with 10^{-6} M EME. D: Nasal mucosa pre-incubated by RPMI with 10^{-5} M EME. All pictures are under the same magnification and the bar represented 100 μ m.

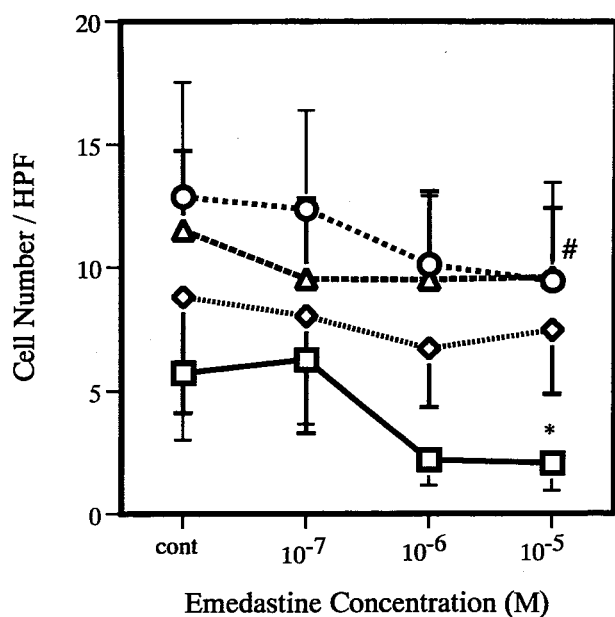


Fig. 6. Inhibition of the number of EG2-positive cells in nasal mucosa at 10 hr after in vitro Ag challenge by the effect of EME in each case, patient (Pt.) No. 1 (□), Pt. No. 2 (◇), Pt. No. 3 (○) and Pt. No. 4 (△). * $P < 0.05$, compared with the control (cont) in Pt. No. 1. # $P < 0.05$, compared with the control (cont) in Pt. No. 3.

DISCUSSION

Analysis of inflammatory cells in epithelial scrapings (5) or lavage fluid (3) obtained from the nasal mucosa is currently the major method of studying allergic reactions of the human nasal mucosa (3). Another approach to investigating the pathophysiology of human nasal allergy is to analyze cytokines (6) and chemical mediators (7) present in nasal mucosal specimens following Ag challenge. There have been many reports on animal models for use in pathophysiological studies of human allergy (8–10). However, the experimental benefit of these animal models is limited by the difference in species with respect to the properties of infiltrating cells and allergic reactions. The adhesive explant culture system used in the present study allows inflammatory cells and structural cells to remain viable in resected nasal mucosal specimens *ex vivo*. It can also preserve the chemotactic response of inflammatory cells to cytokines and adhesion factors as well as the receptor-mediated responses of structural cells (e.g., epithelial cells, glandular cells and vascular endothelial cells) to chemical mediators (11), although any neurally mediated allergic responses and any migrating cells supplied from vessels are lost. In addition, since a piece of nasal mucosa is cultured and fixed onto a Petri dish, the system allows Ag absorption and release of mediators from migrating inflammatory cells.

These absorption and release processes at the nasal mucosa were only through the epithelial surface by preventing the migration of cells and release of mediators from the cut surface covered by clot. The prototype explant culture, on the other hand, allows the allergic reaction to occur from both sides of the tissue section floating in medium. Thus, using the adhesive explant culture, migrating inflammatory cells and mediators released from the cells can be recovered in the medium from the epithelial side of the tissue. This *ex vivo* system may better simulate the *in vivo* allergic reaction of the human nasal mucosa than the prototype tissue culture. This also makes it valid to analyze the kinetics of individual responses. In the present study, the release of mediators during the 30-min immediate response and during the 9.5-hr late response was determined. Many studies have addressed the role of the late response in nasal allergy, but the response has not been clearly defined with respect to its timing relative to Ag challenge; most previous studies used the allergic response from 5 to 9 hr after Ag challenge as the late reaction (12). The 9.5-hr period from 0.5 to 10 hr after Ag challenge assessed in our study certainly covers the late phase of the allergic reaction, but also contains other phases during which inflammatory cells and chemical mediators have negligible activity in the nasal mucosa. However, this period roughly reflects the late phase in view of the negligible inflammatory cell migration and mediator release into the nasal epithelium from 0.5 to 5 hr after Ag challenge according to previous studies (3, 6, 12).

In the present study, culture supernatants were assayed for chemical mediators over 10 hr after Ag challenge, and tissue specimens were subjected to immunohistochemical staining at the completion of the allergic response. Although the kinetics of inflammatory cell migration and chemical mediator release would most desirably be analyzed independently, inflammatory cells were lysed to release chemical mediators, and only mediators, in the culture supernatant were quantified to estimate the nasal allergic response in this pilot study.

Terada et al. (13) suggested that histamine released during the immediate reaction comes from mast cells present in the nasal mucosa and that histamine released during the late phase comes from basophils migrating into the mucosal epithelium. In the present study, pretreatment with EME, an anti-allergic agent with a primary anti-histamine effect, did not suppress histamine release from basophilic cells during the immediate and late phases. This indicates that EME, when applied for only a few hours, blocks histamine receptors but does not inhibit the release of chemical mediators from basophilic cells or the migration of these cells. This is consistent with the clinical experience that adequate efficacy of an anti-

allergic agent can only be obtained by long-term administration and with the *in vitro* finding that azelastine hydrochloride, an antiallergic agent, reduced the expression of cytokine mRNA, but not cytokine production, in cultured nasal mucosal epithelial cells (14). When applied for a longer period during culture or before resection of nasal mucosal specimens, EME may also influence the activation of basophilic cells in the nasal allergic response.

In our preliminary experiment, the ECP level determined in culture supernatant not subjected to freezing/thawing was below the limit of quantification. This finding taken together with the results of the present study indicates that only a small amount of ECP was released by eosinophils and that the ECP level in the culture supernatant reflected the number of eosinophils migrating out of the mucosa during the immediate and late phases. These data were also consistent with findings reported by Terada et al. (13). Migration of eosinophils into the epithelial layer of the nasal mucosa during the immediate phase was not noticeable and was not suppressed by pretreatment with EME. Extensive migration of eosinophils occurred, as reflected by the greater amount of ECP than histamine released during the late versus immediate phase. Migration of eosinophils during the late phase was suppressed by pretreatment with EME at a concentration of 10^{-6} M or more. As suggested previously, EME probably suppressed the migration of eosinophils by mechanisms other than suppression of cytokine production in view of the short duration of pretreatment and the pharmacological profile of the drug. EME has been reported to suppress PAF-induced migration of eosinophils at concentrations of 10^{-8} M or more *in vitro* (15). The same *in vitro* study also demonstrated that EME had no effect on neutrophil migration and that its effect on eosinophil migration was not mediated by histamine H_1 receptors. In the present study, EME inhibited Ag-induced eosinophil migration in the nasal mucosa at concentrations of 10^{-6} M or more. This discrepancy with respect to the effective concentration may be due to methodological differences because the previous study used peripheral blood eosinophils, while the present study observed the migration of tissue eosinophils in the nasal mucosa. Toxic effect of high dose of EME was not detected in the previous study (16), so our study indicates that the inhibitory effect of eosinophil migration and ECP release were by EME itself. Possible mechanisms for the suppressive effect of EME on eosinophil migration include blockade of receptors for eosinophil chemotactic factors including PAF or suppression of their expression. Further studies must be done to clarify this point. One might expect that more eosinophils would remain in the nasal mucosal specimens if release into the culture medi-

um was suppressed. Contrary to this expectation, pretreatment with EME also reduced the number of eosinophils (stained for EG2) in the human nasal mucosa. This suggests that EME might also inhibit the activation of eosinophils after Ag challenge. To verify this presumption, the total number of eosinophils remaining in the nasal mucosa and the percentage of activated eosinophils should be determined accurately by staining for ECP. Further studies are required to obtain sufficient evidence of the suppressive effects of EME on tissue infiltration and activation of eosinophils in the clinical setting of allergy.

The results of the present study also indicate that this *ex vivo* adhesive explant culture system is useful for analysis of the migrating cells of allergic reactions in the immediate and the late phases. It should also be helpful for determining the mechanism of action of anti-allergic agents effective against nasal allergy.

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