

Tissue density and state of activation of eosinophils in the nasal mucosa of allergic and nonallergic rhinopathic patients

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The tissue density of eosinophils and the degree of activation of eosinophils in the nasal mucosa of allergic and nonallergic rhinopathic patients were studied by a combination of the immunogold method, which uses EG2 antibody which binds to the secreted form of eosinophils, and the Luna method. The tissue density of eosinophils and the degree of EG2-positivity were higher in the allergic nasal mucosa than in the nonallergic nasal mucosa. The eosinophils in the tissue were mainly EG2-positive in contrast with those in the vessels. Epithelial shedding was rarely observed and was not related to the tissue density of eosinophils or EG2-positivity. Our results suggest that the eosinophils in the allergic nasal mucosa are activated in the tissue, not in the vessel.

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Eosinophils are widely recognized as being proinflammatory cells and are considered to produce tissue damage (6). Eosinophil activation can now be recognized by the immunohistochemical marker, mouse monoclonal IgG1 antibody, EG2 (18). In the bronchial mucosa of bronchial asthma (1, 3, 4) and in *in vitro* experimental conditions (18), many eosinophils are EG2-positive. The extracellular deposition of the secreted form of ECP has been observed surrounding activated eosinophils and beneath the basal membrane in asthma, and is considered to be related to epithelial damage in bronchi (1, 3).

Eosinophils are numerous in the allergic nasal mucosa and secretions (7, 12, 13). However, epithelial shedding is rarely observed in the allergic nasal mucosa (12). In this study, we used double-staining, immunogold to detect EG2 and Luna staining of the eosinophil (11) to determine eosinophil tissue density and eosinophil activation in four zones of the allergic nasal mucosa, as compared with the nasal mucosa of nonallergic nasal diseases. In addition, to determine whether eosinophils become activated in allergic nasal tissue, we examined the EG2 positivity of eosinophils in the tissue and in the vessels. Moreover, allergic mucosa was examined for extracellular EG2-positive deposition.

Material and methods

Subjects

All patients with allergic or nonallergic nasal disease needed surgical treatment of nasal obstruction.

Twenty patients with nasal allergy were studied; their mean age was 30.0 (range 11–55). The mean duration of their nasal symptoms was 5.1 years (range 2–10). They were positive in an intradermal skin test to house dust (Torii Co., Japan) or had a RAST score higher than 2 to house dust (Greer Lab, USA) or *Dermatophagoides farinae* and had eosinophilia in nasal smear and typical perennial allergic symptoms (repeated attacks of sneezing, watery rhinorrhea, and nasal obstruction). The nasal provocation test to paper disks of house dust (Torii Co.) was positive.

Twenty patients with nonallergic nasal diseases had chronic rhinitis or hypertrophic rhinitis. Their mean age was 32 (range 12–55). They had no attacks of sneezing and watery rhinorrhea, and no eosinophilia in the nasal smear. The mean duration of their nasal symptoms was 4.9 years (range 1–15). Most patients had a negative intradermal skin test to house dust or 0 RAST score to *D. farinae*. In

some patients, the skin test and RAST were not performed.

Materials and tissue processing

Nasal mucosa of the inferior turbinate was obtained by conchotomy under local anesthesia with both spraying of 4% lidocaine containing 0.1% epinephrine and injection of 1% lidocaine into nasal mucosa. The nasal mucosa 10–20 mm from the anterior edge of the inferior turbinate was sectioned. The tissues were fixed with 3.5% neutral buffered formalin for 1 or 2 days. They were dehydrated with a series of ethanol solutions, and then embedded in paraffin-wax. The sections of 3 μ m thickness were dewaxed in xylene and rehydrated.

Staining methods

The sections were washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.2) containing 0.15 M NaCl and 0.01% Triton X-100. They were incubated with 10% heated inactivated normal rabbit serum, and then incubated with the murine monoclonal antihuman eosinophil cationic protein antibody, EG2 (Nichirei Co, Japan), which was diluted 1:10 in PBS containing 0.1% bovine serum albumin and 0.05% NaN₃ (PBS/B/N) for 2 h. Controls for nonspecific staining were incubated with 10 μ g/ml of mouse IgG1 from ascites fluid (MOPC 21) (ICN ImmunoBiologicals, USA). Sections were washed and incubated with biotinylated rabbit antibody to mouse IgG, IgA, and IgM (Nichirei Co) for 1 h. Sections were washed, and then incubated in 1:40 dilution of AuroProbe LM streptavidin (Janssen Biotech NV, Belgium) for 1 h. The samples were washed in PBS and distilled water, and then silver enhancement was performed with IntenSE M (Janssen Biotech NV) for 16 min. Samples were washed, and then were stained with hematoxylin-Biebrich scarlet solution (Luna method (11)). They were dehydrated, and then mounted.

Methods of assessment of slides

The cells with both a nucleus and Luna staining-positive cytoplasm were counted as eosinophils. Cells with one nucleus and abundant cytoplasm which seemed to have phagosomes containing EG2-positive gold particles were rarely observed. These cells were excepted from the counting of eosinophils. The eosinophils which contained no or one gold particle in the cytoplasm were assessed as EG2-negative cells. Individual gold particles were scattered sparsely in all areas of some sections incubated with the negative control or EG2 antibody and could randomly exist over a cell. Eosinophils were counted

in the four zones of nasal mucosa consisting of the epithelial layer, subepithelial layer, glandular area, and vascular area. More than 150 cells were usually counted at more than five areas with an eyepiece graticule (area: 0.0384 mm²) at a magnification of 400. The first area observed was at the right edge of the section. The additional areas were selected at the width of three eye graticules distance. In sections such as nonallergic nasal mucosa with small numbers of eosinophils, eosinophils in all areas of each zone were counted. The number of eosinophils with six gold particles in each cell's cytoplasm was also counted.

Student's two-tailed t-test for comparison of the eosinophil tissue density was performed.

Results

Tissue density of eosinophils

Even the eosinophils with many black gold particles had detectable pink cytoplasmic staining by the Luna method. The distribution of eosinophils was not always the same among different areas in one section. The sections with many eosinophils in the subepithelial layer of allergic nasal mucosa had a tendency to have many eosinophils in the epithelium. On the other hand, the tissue density of eosinophils in the glandular and deep vascular areas did not necessarily correspond with that in the subepithelial layer in one section.

The mean numbers of eosinophils per square millimeter (SD) in allergic nasal mucosa were 50.8 (32.3), 230.3 (179.3), 70.2 (53.7), and 14.6 (14.8) in the epithelium, subepithelial layer, glandular area, and deep vascular area, respectively. In nonallergic nasal mucosa, they were 2.9 (7.6), 24.7 (38.1), 3.0 (8.3), and 0.3 (0.5), respectively. There was a significant difference ($P < 0.01$) between the allergic and nonallergic nasal mucosa in all four portions of the tissues.

EG2-positive eosinophils in allergic nasal mucosa

In the area of the highest tissue density of eosinophils, the allergic subepithelial layer, most eosinophils were positive for EG2 antibody (Fig. 1); the mean percentage of EG2-positive eosinophils was 83.2 (SD: 12.9). Moreover, the eosinophils in the subepithelial layer had more gold particles than those in the other three zones (Fig. 1). The mean percentage of EG2-positive eosinophils in other areas of allergic nasal mucosa (SD) was 70.1 (22.3), 71.1 (15.4), and 66.4 (24.6) in the epithelium, glandular area, and deep vascular area, respectively. As compared with allergic nasal mucosa, the proportion of EG2-positive eosinophils in nonallergic nasal mucosa was lower in all portions.

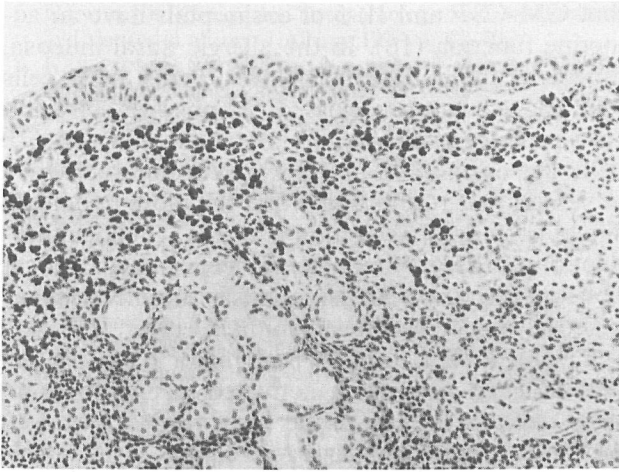


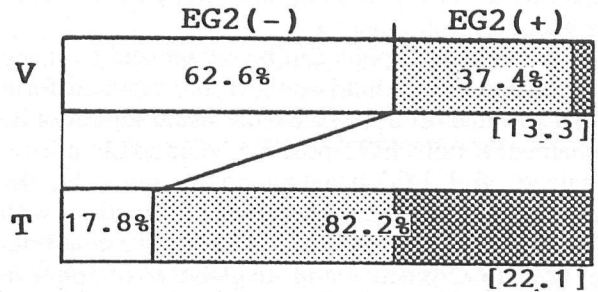
Fig. 1. Allergic nasal mucosa showing large number of eosinophils strongly EG2-positive localized in subepithelial layer. In epithelium and glandular area, eosinophils with a few gold particles have sparsely infiltrated. Shedding was not observed in epithelial layer. Gold immunohistochemistry with EG2 antibody and Luna staining ($\times 90$).

Epithelial shedding was observed in only some areas of all sections in allergic and nonallergic nasal mucosa. In nonallergic specimens with the epithelial shedding, there were no or a few eosinophils in the subepithelial layer under the region of shedding and the other zones. On the other hand, in allergic mucosal specimens, many eosinophils with many gold particles were located under the epithelial desquamation. However, many eosinophils with many gold particles were similarly located under normal epithelium (Fig. 1). Although some gold particles surrounding the eosinophils, indicating extracellular EG2, were observed in small areas of the subepithelial layer in a few allergic mucosal tissues, they did not always exist under the region of epithelial shedding.

EG2-positive eosinophils in tissue and in vessels of allergic nasal mucosa (Fig. 2)

Total numbers of eosinophils in vessels of the glandular and the deep vascular areas were small; therefore these areas are taken together as the deep layer of lamina propria in the study. In the deep layer, eosinophils were observed mainly in cavernous sinuses. In the subepithelial layer, eosinophils existed in capillaries and small vessels. Fourteen nasal mucosa tissues which contained more than 15 eosinophils in vessels at each site of the subepithelial layer and the deep layer of lamina propria were chosen. The percentage of EG2-positive cells in these 14 tissues was confirmed to be similar to the percentage in all 20 tissues. Although most eosinophils in tissues were positive to EG2 antibody, many eosinophils in vessels had no gold particles. Moreover,

Subepithelial layer



Deep layer of lamina propria

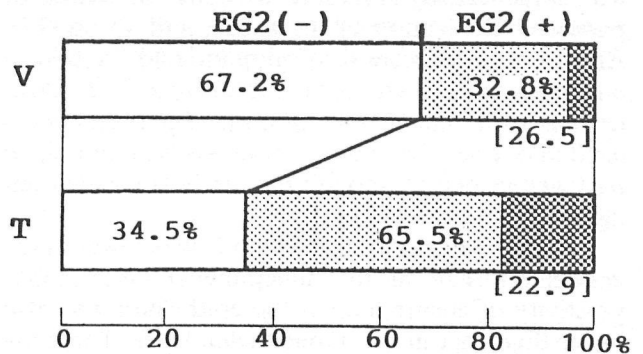


Fig. 2. EG2 positivity of eosinophils compared between eosinophils in tissue and in vessels in allergic nasal mucosa. Percentage of EG2-positive eosinophils is clearly higher in tissue than in vessels at both portions. Area of latticed bar indicates percentage of eosinophils with strong reaction to EG2 antibody (more than six gold particles in cytoplasm). Mean percentage of EG2-positive eosinophils in tissue and rate of strong reaction to EG2 antibody are higher in subepithelial layer, where many eosinophils are located, than in deep layer. V and T show vessels and tissues, respectively ($n = 14$). Square brackets indicate SD.

many eosinophils in tissue, especially in the subepithelial layer, had many gold particles in their cytoplasm. On the other hand, EG2-positive eosinophils located in vessels had only a few gold particles.

Discussion

The increased number of eosinophils in nasal scrapings and the increased amount of eosinophil granular proteins in nasal washing fluid suggest a relationship with nasal hypersensitivity (8, 10) or nasal symptoms such as nasal obstruction (19).

Previous observations on small biopsy specimens have also shown many eosinophils located in the allergic nasal subepithelial layer (7, 12). Analysis of eosinophil tissue density among the zones of the nasal mucosa was not performed. Since the distribution of eosinophils in nasal mucosa is not always even, even in one section, our large materials obtained from conchotomy are more suitable to measure the mean number of eosinophils in the lamina

propria and epithelium. We confirmed differences in the tissue density of eosinophils among the zones in the allergic nasal mucosa.

Activated eosinophils can be recognized by using EG2 antibody that binds only to the secreted form of intracellular ECP (18). By the usual immunohistochemistry, only EG2-positive eosinophils are recognized, and EG2-negative ones cannot be detected. By combining the immunogold method with EG2 antibody and Luna staining (11), we could detect both EG2-positive and -negative eosinophils in one section. It has been reported that the number of labeled immunogold particles in the ultrathin section was influenced by several factors such as antibody, particle size, fixation of the tissue, and so on (17). Although the procedure of immunogold method in our study was at the light microscopic level, eosinophils with many, some, or no gold particles coexisted in one section (Fig. 1). Numerous immunogold particles in one eosinophil may indicate a high antigenicity to EG2 antibody.

The tissue density of eosinophils was lower in the epithelium than in the subepithelial layer. EG2-positivity of eosinophils in the epithelium was also lower than that in the subepithelial layer. There are two possible explanations to consider. First, the chemoattractant of eosinophils in nasal secretions derived from nasal surface cells may be strong, and most eosinophils do not remain in the epithelium but migrate quickly to nasal secretion without being activated. Second, EG2 antibody binds to the secreted form of eosinophil cationic protein (ECP) (18). If eosinophils in nasal epithelium release ECP or degranulate completely, the antigenicity to ECP in the granules of eosinophils would disappear. There would not be a positive reaction product to EG2 antibody in these eosinophils.

It has been reported that 30% of eosinophils in the peripheral blood in patients with nasal allergy are hypodense (5). Although the relationship between hypodense eosinophils and EG2-positive eosinophils is unknown, both findings indicate activation of eosinophils. EG2-positive eosinophils in vessels in allergic nasal mucosa were about 30% in our observation, and this value coincides with the percentage of hypodense eosinophils in peripheral blood (5). Most EG2-positive eosinophils in the vessels of allergic nasal mucosa were weakly positive. On the other hand, the eosinophils in tissues showed a high rate of EG2-positivity and strong reactivity to EG2 antibody. It has been reported that interleukin (IL)-5 is an eosinophil chemoattractant (15). IL-5, IL-3, and granulocyte/macrophage colony-stimulating factor (GM-CSF) are known to prolong survival and activation of eosinophils *in vitro* (15). These cytokines can be produced from activated T cells, mast cells (15), and eosinophils (9). It is also suggested

that GM-CSF and IL-5 of eosinophils have an autocrine function (16). In the allergic nasal mucosa, especially the subepithelial layer, many mast cells (14) and eosinophils exist. CD4-positive cells and IL-2-receptor-positive cells are also observed (2). Although eosinophils may begin to become activated in vessels, our results show that the more important site of eosinophil activation is tissue. The local production of these cytokines may determine the location and activation of eosinophils in the allergic nasal mucosa, especially the subepithelial layer.

Chemical mediators such as major basic protein and ECP released from activated eosinophils and their deposits in the tissue have been thought to produce the epithelial damage in asthma (1, 3). However, it had been reported that the structure of allergic mucosal epithelium does not show a severe abnormality similar to the shedding in asthma (12). Even in this study, shedding was observed in only a few areas of both allergic and nonallergic nasal mucosa, and many activated eosinophils did not necessarily localize in relation to the shedding. Moreover, clusters of gold particles could not be observed in the allergic and nonallergic nasal mucosa except in the cytoplasm of eosinophils. The function of eosinophils is considered to include tissue toxicity. At least, in nasal allergy, eosinophils did not seem to be related to epithelial shedding.

In conclusion, many eosinophils exist in the allergic nasal mucosa, especially in the subepithelial layer. Most of them are activated in the tissue, not in the vessels.

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