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Originals

ECP Levels in Nasal Mucus Depend on Eosinophil Collapse

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SUMMARY

Objective : Degranulation of eosinophils is believed to cause epithelial failure. Some researchers have believed that degranulation of eosinophils occurs in the form of piecemeal degranulation (PMD), while others have reported that breakdown of the eosinophil cell membrane causes degranulation. In this study, I examined by which form the eosinophil granule proteins in nasal mucus are mainly released from eosinophils.

Methods : Nasal mucus collected by suction were observed by electron microscopically. Total eosinophils, collapsed eosinophils, and vacuolated granules were counted. Destruction of eosinophil cell membrane was classified into 4 ranks by degree of failure of the cell membrane. Granule vacuolation was classified into 3 ranks based on granule properties. A destruction index was defined as the product of the sum of granule vacuolation score and the score for destruction of eosinophil cell membrane. The ECP level in lavage fluid from the nasal cavity was also measured.

Results : The ECP level in lavage fluid and score for destruction of eosinophil cell membrane were significantly correlated ($R = 0.813$, $p = 0.0077$), while ECP level and destruction index which was derived from both the destruction of cell membrane and granule vacuolation exhibited an even stronger correlation ($R = 0.988$, $p < 0.0001$).

Conclusion : The eosinophil granule proteins in nasal mucus were released mainly from collapsed eosinophils.

Key Words : eosinophil, degranulation, ECP, nasal mucus, destruction of cell membrane, destruction index

INTRODUCTION

Eosinophils can be induced by certain cytokines and chemokines to exhibit chemotactic migration to local sites of allergic inflammation. It is thought that degranulation of eosinophils which migrate to the nasal mucous membranes causes epithelial failure. Ultrastructural evidence suggests the existence of at least two degranulation events in eosinophils : piecemeal degranulation (PMD), in which granular content is released from intracellular granules leaving more or less empty granules in the intact cell [1], and eosinophil cytolysis, in which the cell membrane ruptures, causing the release of free eosinophil granules. Although there is general agreement regarding the occurrence of PMD, the ultrastructural patterns of granules during this event as it proceeds in the airway mucosa remain largely unexplored. Watanabe et al [2] reported in 1977 that breakdown of eosinophil cell membranes caused degranulation of eosinophils, and Persson and colleagues [3, 4] recently published a large number of re-

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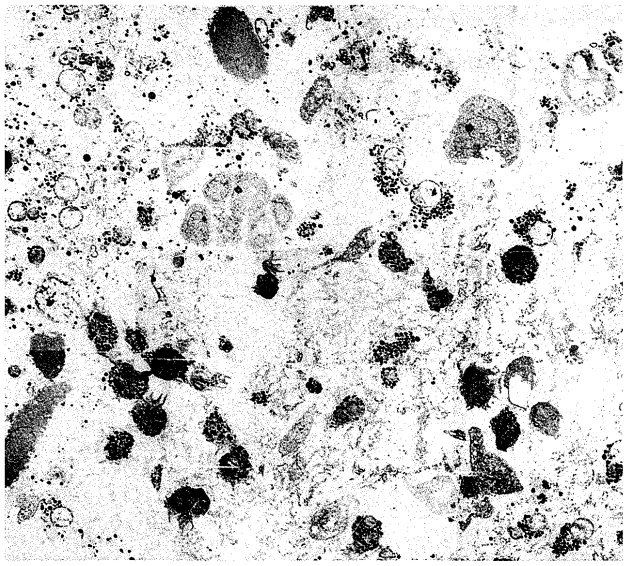

Fig. 1 Complete photograph of 117μm square hole obtained from about 20-25 electron micrographs.

ports on eosinophil collapse in local sites of allergic inflammation. In addition, Watanabe et al[5] found that the rate of collapse of eosinophils in nasal mucosa and epithelial shedding were significantly correlated. It has been thought that eosinophils which migrate to the nasal mucus also collapse, and it has been confirmed that the severity of nasal symptoms and the rate of collapse of eosinophils in nasal mucus are significantly correlated[6]. It has thus been shown that the eosinophils which migrated to the nasal mucosa partially collapsed in nasal epithelium, and subsequently collapsed in large numbers in nasal mucus. Nevertheless, the events of PMD or cytolysis responsible for degranulation in nasal mucus will remain unclear until the granule protein levels in nasal mucus and morphological changes of eosinophils are examined. The rate of collapse of eosinophils is not correlated with granule protein level in mucus if PMD is occurring at a high rate.

In this study, we examined the eosinophil granule protein in nasal mucus released chiefly from collapsed eosinophils.

MATERIALS AND METHODS

Examination was performed in 9 patients, including 7 females and 2 males who ranged in age from 21 to 52 years, who had symptoms of nasal allergy, and had eosinophils in nasal mucus. None of the patients had received anti-allergic drugs for at least 2 months before collection of nasal mucus. Nasal mucus was first collected by suction from the left nasal cavity, fixed for 2 hours by immersion in ice-cold 2% glutaraldehyde buffered with 0.1 M/L cacodylate, pH 7.3, and then washed three times with 0.1 M/L cacodylate buffer containing 8% sucrose, pH 7.3. The specimens were then post-fixed for 40 minutes in 2% osmium tetroxide and embedded in Epon 812. Ultrathin cross-sections were prepared with a microtome (RMC MT 6000XL), and stuck on a mesh grid with 117 μm square holes. Complete photographs of each hole were obtained from 20-25 electron micrographs taken at 3000-fold magnification (Fig 1). Complete photographs of 5 randomly selected holes were prepared for every sample. All eosinophils in the 5 holes were counted, and eosinophil number was scored using the criteria listed in the Table. Destruction of eosinophil cell membrane was classified into 4 ranks from 0 to 3 based on degree of failure of the cell membrane. The individual scores for destruction of eosinophil cell membrane in the 5 holes were added to obtain a total destruction score. Granule vacuolation was classified into 3 ranks from 0 to 2 based on granule properties. The destruction index was defined as the product of the sum total of granule vacuolation score and the score for destruction of the eosinophil cell membrane (when the score for destruction of eosinophil cell membrane was 0, the destruction index was considered 0 regardless of granule vacuolation score). For example, for the eosinophil shown by A in Fig 2, the score for destruction of eosinophil cell membrane is 0 because the cell membrane is intact

Table 1 Scores for degree of destruction of eosinophil cell membrane and granule vacuolation, and definition of the destruction index.

Score for destruction of eosinophil cell membrane
0 : Intact cell membrane.
1 : Less than 50% destruction of cell membrane.
2 : 50% – 75% destruction of cell membrane.
3 : More than 75% destruction of cell membrane.
Granule vacuolation score
0 : Normal granules
1 : Filled with coarse granules
2 : Vacuolation
Destruction index
(sum of granule vacuolation score) × (score for destruction of eosinophil cell membrane)

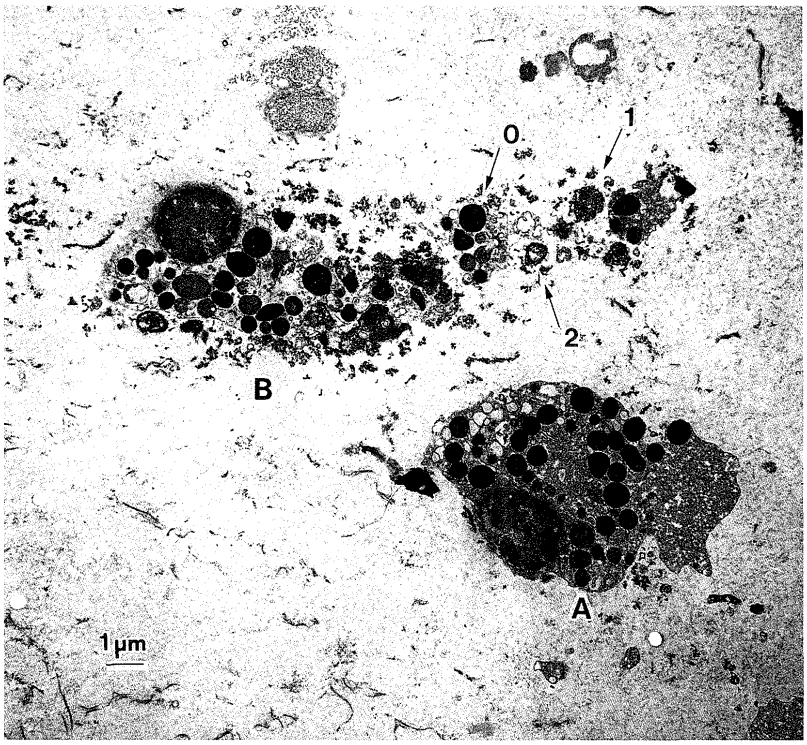

Fig. 2 A, Eosinophil with intact cell membrane. B, Eosinophil with cytolysis of cell membrane (Destruction score 3).
arrow 0 : Granule vacuolation score 0
arrow 1 : Granule vacuolation score 1
arrow 2 : Granule vacuolation score 2

over its entire circumference, and therefore the destruction index is also 0. For the eosinophil shown by B, on the other hand, the score for destruction of eosinophil cell membrane is 3 because the cell membrane is missing over the entire circumference (more than 75% destruction of cell membrane) and this eosinophil contains granules with various properties. Since 21 granules are scored 0, 6 granules are scored 1, and 2 granules are scored 2, the granule vacuolation score is $(0 \times 21) + (1 \times 6) + (2 \times 2) = 10$. Therefore, the destruction index for the eosinophil identified by B is $3 \times 10 = 30$. The sum of the individual destruction indices for the 5 holes was considered the total destruction index. The sum total of score for destruction of eosinophil cell membrane and destruction index for all eosinophils in 5 holes was calculated.

The right nasal cavity was lavaged with 30 ml physiological salt solution, using pumps that discharged 3 ml per press. The fluid obtained with 10 lavages performed in this fashion was collected in a tray, centrifuged at 1500 g for 10 minutes, and the ECP in the supernatant measured by Venge's method[7] using a Pharmacia ECP-RIA kit. The correlations between ECP level in nasal mucus and total number of eosinophils in nasal mucus, total score for destruction of eosinophil cell membrane, and total destruction index were examined.

RESULTS

Two types of eosinophils were observed in nasal mucus. One had an intact cell membrane (Fig 2, A) while the other had a cell membrane that exhibited cytolysis (Fig 2, B). These types of eosinophils had different levels of granule vacuoles. The ECP level in nasal lavage fluid and total number of eosinophils in 5 holes were significantly correlated ($R = 0.701$, $p = 0.0353$, Fig 3), ECP level in nasal lavage fluid and total score for destruction of eosinophil cell membrane were more significantly correlated ($R = 0.813$, $p = 0.0077$, Fig 4), while ECP level in nasal lavage fluid and total destruction index exhibited the strongest correlation ($R = 0.988$, $p < 0.0001$, Fig 5).

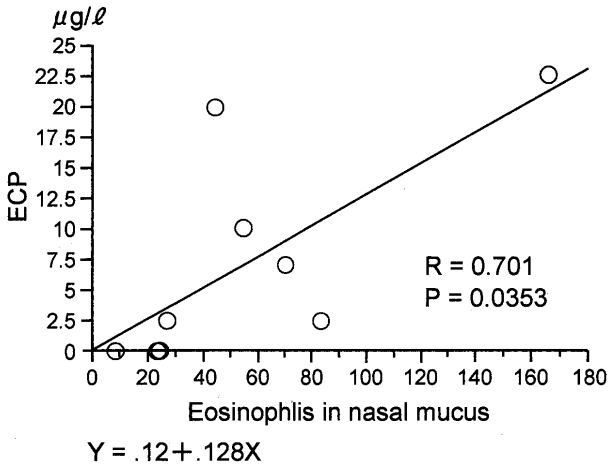

Fig. 3 ECP level in nasal lavage fluid and total number of eosinophils exhibited a mild correlation (R = 0.701, p = 0.0353).

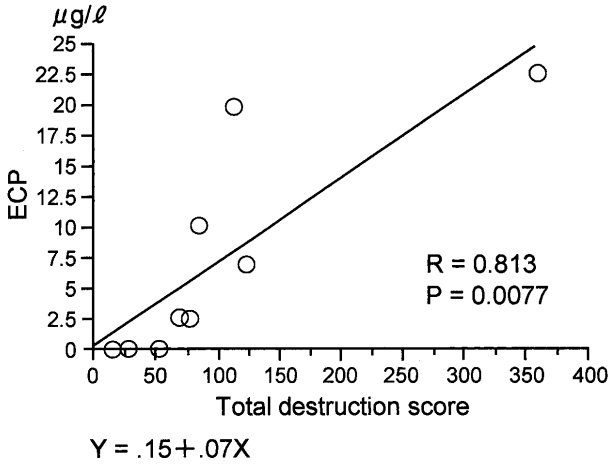

Fig. 4 ECP level in nasal lavage fluid and total destruction score of eosinophil cell membrane exhibited a strong correlation (R = 0.813, p = 0.0077).

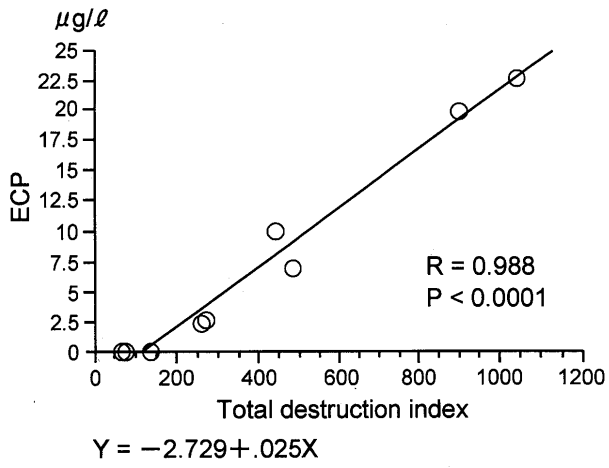

Fig. 5 ECP level in nasal lavage fluid and total destruction index exhibited the strongest correlation (R = 0.988, p < 0.0001).

DISCUSSION

Eosinophil granule proteins are known to cause epithelial failure. There have been many recent reports that eosinophils degranulate in vivo as a result of cytolysis [3~6, 9]. Although, not all researchers agree with this conclusion. It has been reported that IL-5 caused eosinophil degranulation [10]. Although degranulation was not induced by normal IL-5 levels in nasal mucus, a large amount of ECP was detected in it [8]. It thus appears that some agent or process other than IL-5, for example cytolysis, induces degranulation in vivo.

Ponikau et al [11,12] insisted that cytotoxic protein was released from degenerated and lysed eosinophils and injured the epithelium, mainly from the outside (luminal side), and that ECP level in nasal mucus, which exists on the luminal side, is therefore important. However, there have been few previous reports on the granule proteins in nasal mucus released by cytolysis. Demonstration of a significant correlation between eosinophil cytolysis and ECP level in nasal lavage fluid would thus be of great importance. It was found in this study that ECP level increased to the greatest extent when eosinophils with many vacuolated granules were destroyed.

Erjefalt et al [9] reported that eosinophils in nasal mucosa that exhibited cytolysis had few vacuolated granules, although eosinophils with intact cell membrane had many such granules. The results of the present study differ slightly from these findings, since many vacuolated granules were found in cytolytic eosinophils. The eosinophils observed in this study were obtained from nasal mucus and not mucosa. The granules from lysed eosinophils may vacuolate as soon as they are released into the nasal mucus, which features a severe extracorporeal environment. The possibility that granule vacuolation occurs after cell failure thus cannot be ruled out. At any rate it could be shown that granule proteins are not released into the extracellular space if the cell membrane does not collapse, even if the granule vacuolation score is high. Watanabe and colleagues have reported that EPO, one of the granule proteins in eosinophils, would not have been released

from eosinophils in nasal mucosa if the cell membrane of the eosinophil had remained intact, although EPO was released into the cytoplasm when eosinophils were activated[2, 5, 6]. The present study supports these findings. It was found in this study that ECP level in nasal mucus and degree of eosinophil collapse were significantly correlated, indicating that extracellular release of eosinophil granule proteins was a result of cytolysis. It thus appears that the degranulation of eosinophils not only in nasal mucosa but in nasal mucus depends on factors causing cytolysis.

In asthma, a great proportion of eosinophils infiltrating the bronchial mucosa are of low density, and these eosinophils are increased in number even in the peripheral blood in association with asthma attacks[13]. One of the morphological characteristics of hypodense eosinophils in BAL fluid on electron microscopic examination is cellular vacuoles[14], which appear similar to those noted just prior to cytolysis. The eosinophils in nasal lavage fluid are similar to those in BAL. Many hypodense eosinophils were also observed in nasal lavage fluid. It may thus be that activated eosinophils and hypodense eosinophils are the same. Since hypodense eosinophils may be fragile, activated eosinophils may also readily undergo cytolysis. Aalbers et al[15] reported the finding of lack of correlation between increase in amount of ECP and number of EG2+ cells in bronchial lavage following allergen challenge. If they had counted the numbers of collapsed EG2+ cells, their results would have been different. Misu[16] reported that serum ECP was detected one hour after blood collection as a result of eosinophil collapse in test tubes. He speculated that the eosinophils which easily collapsed in test tubes will collapse as soon as they have migrated into the nasal mucus.

It was reported in 2000[17] that antibody to IL-5 lowered blood and sputum eosinophil counts, but had no significant effect on late asthmatic response or airway hyperresponsiveness to histamine. This finding suggested the possibility that eosinophils do not play a major role in the pathogenesis of allergic diseases. It may be that eosinophils do not in fact play major roles in allergic diseases. However, since the possibility that eosinophil granule proteins have injurious effects on epithelial cells cannot be ruled out, it is possible that the epithelium is affected by eosinophil granule proteins. If eosinophil granule proteins could be rapidly removed from the nasal cavity, they would not severely affect the nasal epithelium. The viscosity of mucus may be of key importance, since collapsed eosinophils confined to the viscid nasal mucus are difficult to remove from the nasal cavity.

The findings obtained in this study indicate that, in nasal mucus, granule proteins are released from activated eosinophils with broken cell membranes, and that more granule proteins are released when eosinophils with many vacuolated granules collapse.

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REFERENCES

1) Dvorak AM, Furitso T, Letourneau L, Ishizaka T, Ackerman L. : Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures with human recombinant interleukin-5. I . Piecemeal degranulation of specific granules and distribution of Charcot-Leiden crystal protein. Am J Pathol, **138** : 69-82, 1997.

2) Watanabe K, Hasegawa M, Saito Y, Takayama S. : Eosinophilic leucocytes in nasal allergy-Movement of enzymes. Clin Allergy, **7** : 263-271, 1977.

3) Persson CG, Erjefalt JS. : Eosinophil lysis and free granules : an in vivo paradigm for cell activation and drug development. Trend Pharmacol Sci, **18** : 117-23, 1997.

4) Andersson M, Greiff L, Erjefalt JS, Svensson C, Persson CG. : Allergic and infectious rhinitis-similarities and disparities. Allergy Clin Immunol Int, **11** : 11-5, 1999.

5) Watanabe K, Kiuna C, Misu T. : Degranulation of human eosinophils in nasal allergy. Jpn J Allergol, **48** : 500-506, 1999.

6) Watanabe K, Misu T, Inoue S, Edamatsu H. : Cytolysis of eosinophils in nasal secretions. Ann Otol Rhinol Laryngol, **112** : 169-173, 2003.

7) Venge P, Roxin LE, Olsson I. : Radioimmunoassay of human eosinophil cationic protein. Br J Haematol, **37** : 331-335, 1977.

8) Terada N, Konno A, Shirotori K, et al : In human nasal mucosa, interleukin-5 accumulates and degranulates eosinophils. Rhinology, Suppl **14** : 57-61, 1992.
9) Erjefalt JS, Greiff L, Andersson M, Matsson E, Petersen H, Linden M, Ansari T, Jeffery PK, Persson CGA. : Allergen-induced eosinophil cytolysis is a primary mechanism for granule protein release in human upper air way. Am J Resp Crit Care Med, **160** : 304-312, 1999.
10) Fujisawa T, Abu-Chazaleh, R, Kita H, Sanderson CJ, Gleich GJ. : Regulatory effect of cytokines on eosinophil degranulation. J Immunol, **144** : 642-646, 1990.
11) Ponikau JU, Sherris DA, Kern EB, Homburger HA, Frigas E, Gaffey TA, Robert CD. : The diagnosis and incidence of allergic fungal sinusitis. Mayo Clin Proc, **74** : 877-884, 1999.
12) Ponikau JU, Sherris DA, Kephart GM, Kern EB, Congdon DJ, Adolphson CR, Springett MJ, Gleich GJ, Kita H. : Striking deposition of toxic eosinophil major basic protein in mucus : implications for chronic rhinosinusitis. J Allergy Clin Immunol, **116** : 362-369, 2005.
13) Fukuda T, Dunnette SL, Reed CE, Ackerman SJ, Peters MS, Gleich GJ. : Increased numbers of hypodense eosinophils in the blood of patients with bronchial asthma. Am Rev Respir Dis, **132** : 981-985, 1985.
14) Sedgwick JB, Calhoun WJ, Vrtis RF, Bates ME, McAllister PK, Busse WW. : Comparison of airway and blood eosinophil function after in vivo antigen challenge. J Immunol, **149** : 3710-3718, 1992.
15) Aalbers R, De Monchy JGR, Kauffman HF, Smith M, Hoekstra Y, Vrugt B, Timens W. : Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. Eur Respir J, **6** : 840-847, 1993.
16) Misu T. : The difference between the serum ECP and blood plasma ECP in nasal allergy. Dokkyo J Med Sci, **31** : 57-63, 2004.
17) Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ. : Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness and the late asthmatic response. Lancet, **356** : 2144-2148, 2000.