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Injurious effect of eosinophil extract on the human nasal mucosa

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SUMMARY

Eosinophils are frequently associated with the manifestation of nasal allergy in the nasal mucosa and nasal secretion. The role of eosinophils in hypersensitivity diseases, however, is still obscure, whether it protects or damages tissues and activates mast cells.

The effects of two kinds of human eosinophil extract (biological and physical extracts) on human nasal mucosa by applying them in nasal provocation test and cilia beating test, and also in tracheal ring incubation and skin test in the guinea-pig were measured.

The results of the study suggest that eosinophil major basic protein and other protein components may produce damage to the function of human nasal mucosa and tracheal mucosa of the guinea-pig.

INTRODUCTION

Eosinophils increase in the blood and the involved sites in Type 1 allergy. Their role in allergy however is still unknown. Recently injurious effect of eosinophils in allergic reaction has been proposed and strongly emphasized by Gleich et al. (1979), Kay (1985) and so on. Intensive studies by Gleich's group provided evidence supporting the fact that MBP (major basic protein), the major protein component in the core of eosinophils specific granules, is the most important chemical substance for tissue damage of the airway and skin. Namely, MBP is found in sputum (Frigas et al., 1981) and tracheal mucosa in patients with asthma (Filley et al., 1982) and in the dermis of urticaria (Peters et al., 1983). Tissues such as guinea-pig trachea, spleen, skin etc., when incubated with MBP, were damaged (Gleich et al., 1979).

In spite of the above evidence, questions arise as to whether or not 1) it is also true in allergic rhinitis as well as in asthma and urticaria; 2) MBP is actually released and functions in the allergic mucosa; 3) MBP is the only one chemical substance from eosinophil granules to injure the nasal mucosa.

Because no evidence has been presented on these problems, effects of MBP have been studied on the trachea but not on the nasal mucosa. Gleich et al. used in their studies with MBP which was extracted physically but not biologically from eosinophil granules. Different kinds of proteins other than MBP are contained in

eosinophils (peroxidase, eosinophil cation protein, eosinophil-derived neurotoxin, Charcot-Leyden crystal protein, and granule-associated enzymes etc.), but the effect of MBP is very much emphasized without any comparative study. The purpose of the present study is to answer these questions using extracts from eosinophils by two kinds of methods, different fraction of extract and different target tissues.

MATERIALS AND METHODS

1. *Collection of eosinophils and extraction of eosinophil contents*

Heparinized venous blood was taken from patients with eosinophilia of over 10% of leukocytes, and the eosinophil-rich fractions of over 90% purity were obtained by the Gartner's method (1980). Eosinophils thus collected, 4×10^7 in number, were added in the test tube with distilled water and destroyed completely by repeated freezing and thawing. After centrifugation at 5,000 g for 5 min, the supernatant was collected and freeze-dried for storing. This eosinophil crude extract was resolved in phosphate-buffered saline (PBS) pH 7.2 in different concentrations before use. This was refered to as extract A (Ext A). Another 4×10^7 eosinophils were processed by freezing and thawing in pH 5, 0.05M sodium-acetate-acetic acid buffer for gel filtration. The extract obtained was called as Ext A'.

Eosinophils, 4×10^7 in number, were also incubated with 1 : 1 IgG, anti-IgG immune complex which was made as described previously (Okuda et al., 1983); briefly, human IgG in concentration of 0.8 mg/ml (Miles Scientific, USA) and rabbit anti-human IgG with antibody titer of 0.8 mg/ml (Miles Scientific, USA) were equally mixed, and stood at 37 °C for 1 hr and then at 4 °C overnight. Therefore, precipitate of immune complex produced was washed with PBS by repeated centrifugation at 5,000 g at 4 °C for 30 min; protein amount was adjusted to 1 mg/ml. After incubation at 37 °C for 1 hr with gentle shaking, supernatant which contained substance released from eosinophils by stimulation of immune complex was seperated from cell component. We refered to this as biological extract (Ext B). Cells in precipitate were examined by trypan blue dye exclusion test and processed for electron microscopy.

2. *Electrophoresis*

a. After reduction with dithiothreitol and alkylation with iodoacetamide, the molecular weight of proteins was estimated by electrophoresis on Laemmli sodium dodecylsulfate (SDS) discontinuous buffer system with 2.5% acrylamide 0.1% SDS as stacking gel and 12.5% acrylamide 0.1% SDS as resolving gel described by Hames (1982).

b. The isoelectric point of the eosinophil proteins was determined by electric focusing in polyacrylamide gels using ampholine (LKB, Bromma, Sweden) with a pH gradient of 3.5–10 and 9M urea (Lan and Chrambach, 1982).

c. Two dimensional gel electrophoresis was performed to determine both the molecular weight and isoelectric point of proteins by the procedure described by Sinclair and Rickwood (1982) as follows: briefly, with ampholine pH 3.5–10 gel as first dimension and Laemmli SDS slab gel as second dimension, they were connected by agarose gel for electrophoresis.

3. *Gel filtration*

Fractionation of Ext A' in pH 5, 0.05M sodium acetate-acetic acid buffer was performed on 1.2 x 50 cm column of Sephadex G-50 (Pharmacia Fine Chemicals, Sweden) at a speed of 5 ml/hr and 2 ml/fractionation. The absorbance of each fraction was checked at 225 nm with spectrophtometer (Gleich et al., 1973).

4. *Peroxidase determination*

The determination of peroxidase was carried out by the guaiacol method of Jermyn and Thomas (1954). The optical density of the above reaction product was measured at 400 nm wave length by means of a spectrophotometer. Lactoperoxidase (Sigma, USA) was used as standard.

5. *Protein determination*

Protein amount of eosinophil extract was determined by the Lowry method (1951).

6. *Determination of lactic dehydrogenase (LDH)*

Eosinophils were incubated with IgG immune complex and centrifuged as described above. Then, LHD in the supernatant was determined by the King's method (1959).

7. *Electron microscopical study*

Precipitates from the physical destruction and immune complex incubation of eosinophils were fixed in cold 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2 hr. After overnight washing in the same buffer, the specimens were postfixed in 2% osmic acid for 2 hr, dehydrated with a series of increasing concentrations of ethanol, and embedded in Quetol 812. Ultrathin sections were doubly stained with uranyl acetate and lead nitrate and observed with a Hitachi H-500H electron microscope.

8. *Responses of guinea-pig skin and trachea to extract*

After shaving of a Hartley guinea-pig 250 g in body weight, the abdominal skin was subcutaneously injected by 0.02 ml of Ext A with concentrations of 0.1, 1.0, 10 mg/ml and was observed for 24 hr. After sacrifice, the specimens of tracheal rings with 8 mm x 2 mm were incubated with 0.1, 1.0, 10, 100 mg/ml at 37 °C under 100% humidity for 1 hr. The skin and tracheal rings were then thin-sectioned and hematoxylin-eosin stained for histological examination.

9. *Nasal provocation test*

0.01 ml of Ext A in concentrations of 0.1, 1.0, 10 mg/ml, soaked in the paper disc, were applied to the bilateral inferior turbinates of 12 nasal allergic and 5 non-allergic patients and nasal response produced were observed for 10 min.

10. *Inhibition of nasal cilia beating*

Three pieces of nasal scrapings from the inferior turbinates of each allergic and non-allergic patients were placed on three Fuchs-Rosenthal counting plates immediately after collection. Then, 0.1 ml of the following solutions were dropped on specimens, covered and sealed by a thin small glass at 37 °C, 100% humidity. The first set of solutions were 0.1 and 1.0 mg/ml of Ext A in PBS, 1 mg/ml of human serum albumin in PBS (control) and the second set of solutions were 0.1 mg/ml of 1st peak and 0.1 mg/ml of 2nd peak fractions from gel filtration of Ext A', and pH8 acetate-Tris HC1 buffer solution (control). The cilia beating was observed under the phase contrast microscope until complete stop.

RESULTS

1. From each 4×10^7 eosinophils, were obtained 7.4 mg of Ext A, 5.4 mg of Ext A' and 3.4 mg of Ext B in protein amount.
2. After physical destruction, most eosinophils were destroyed remaining some cell debris with the nucleus. On the other hand, the eosinophils after IgG immune complex incubation showed that the specific granules were degranulated but the cell membrane and cytoplasm were almost intact in ultrastructure (Figure 1) and viable in dye exclusion test.

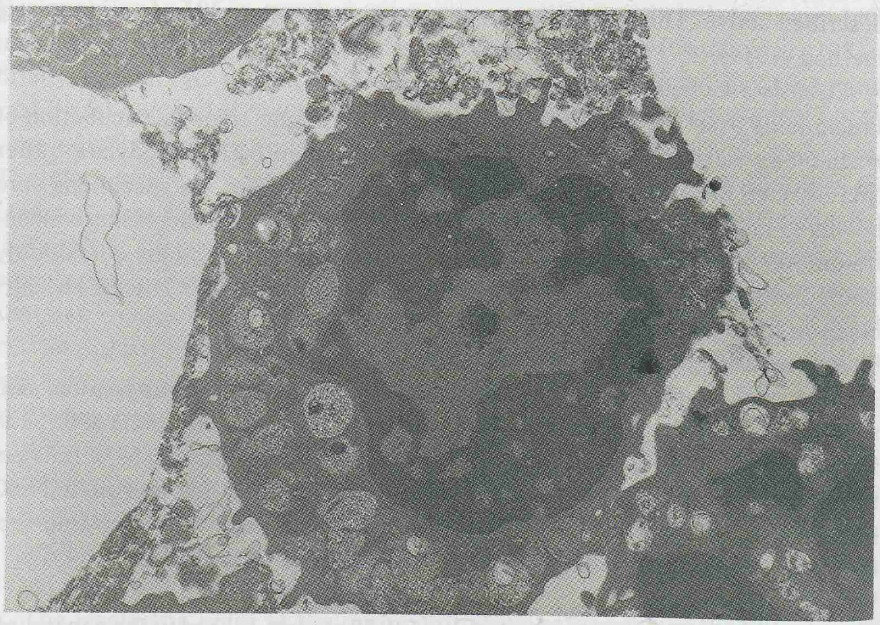

Figure 1. Degranulation of eosinophil with intact cell membrane and cytoplasm when incubated with IgG immune complex.

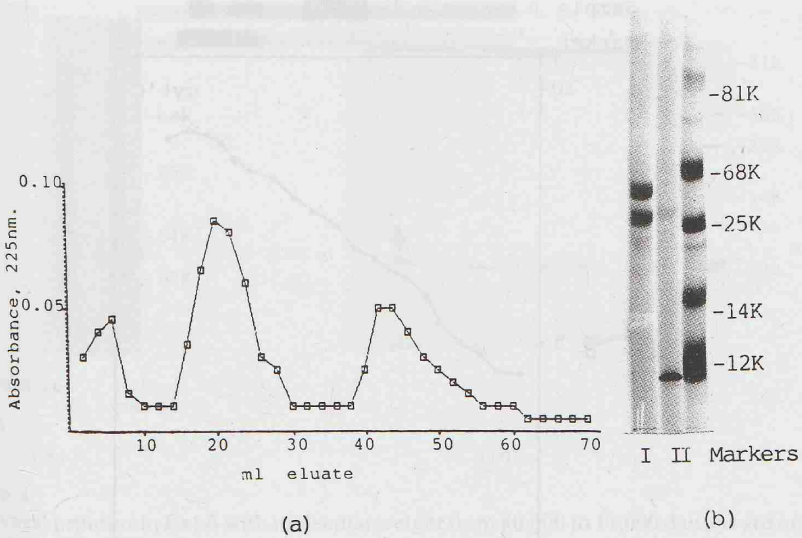

Figure 2. Analysis of Ext A' from disrupted eosinophils on Sephadex G-50 gel infiltration.

a. Eosinophils were disrupted with freezing and thawing in pH5, 0.05M sodium acetate-acetic acid buffer and which was fractionationed on 1.2 x 50 cm column of Sephadex G-50 equilibrated with pH5 acetate buffer containing 0.4M NaCl.

b. The fractions from the 1st and 2nd peaks were adjusted to pH8 with 1M Tris HCl and electrophoresed with Laemmli SDS polyacrylamide gel. The gel rods, right, marker proteins; middle, fractions of the 2nd peak; left, fractions of the 1st peak. Molecular weight marker proteins: creatine-kinase, 81,000 daltons; bovine serum albumin, 68,000 daltons; chymotrypsinogen, 25,700 daltons; lysozyme, 14,300 daltons; cytochrome C, 12,400 daltons.

3. Peroxidase determination. 30 units of peroxidase per 1 mg of Ext B and 6.6 units per 1 mg protein from the fractions of the 1st peak by gel filtration of Ext A' were noted, but not in the fractions of 2nd peak.

4. No LDH was detected in the supernatant from eosinophil-immune complex incubation.

5. Electrophoresis.

a. The disrupted eosinophils (Ext A') analyzed by gel filtration with Sephadex G-50 and Laemmli SDS polyacrylamide electrophoresis could separate proteins with molecular weigth over 20,000 daltons (fractions of the 1st peak) from proteins under 20,000 daltons (fractions of the 2nd peak) which contained a protein with molecular weight about 11,000 daltons (Figure 2).

b. The Ext B contained proteins including a basic (pH10) and low molecular weight one (11,000 daltons) and other large proteins shown in isoelectric focussing, Laemmli SDS gel and 2-dimensional electrophoresis (Figures 3,4).

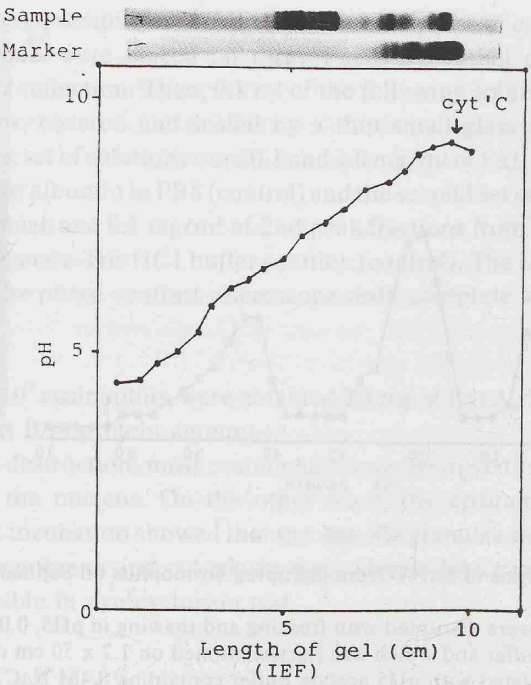

Figure 3. Isoelectric focusing (IEF) of Ext B using 5% polyacrylamide gel in urea and ampholine with pH gradients of 3.5–10. The relative pI of the extract (upper rod) in relation to the pI marker protein, cytochrome C pI 10.6 (lower rod) is shown.

6. In guinea-pig skin test by the subcutaneous injection of Ext A, no wheal or flare was noted during observation, and slight inflammatory cell infiltration was found in 10 mg/ml injected site in histological examination (Figure 5).
7. In guinea-pig tracheal incubation with 100 mg/ml Ext A, the lining epithelium was irregular, surface uneven, with mild hyperemia, desquamation and cell infiltration in histological examination (Figure 6).
8. In human nasal provocation test with Ext A, out of 12 nasal allergic patients, there was none but one patient who had symptoms such as sneezing and rhinorrhea and no symptom at all in the control group.
9a. In human nasal cilia beating test with Ext A, there was a significant difference between control and eosinophil extract, and a tendency with dose-dependence effect. With the control, the cilia beating activity was stronger than the nasal allergic group ($p < 0.01$), and the effect of the extract was dose-dependend ($p < 0.01$). In the nasal allergic group, the cilia beating was weak and the difference between the two concentrations was not so significant ($p > 0.05$) (Table 1).

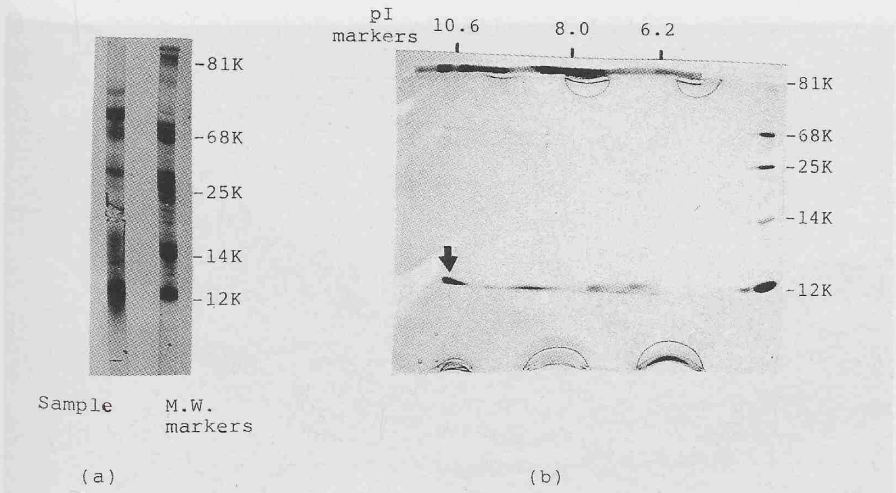

Figure 4.

a. Several proteins in Ext B with molecular weight from 80,000 to 11,000 daltons are noted in this SDS polyacrylamide gel. The molecular weight marker proteins are the same as in Figure 2.

b. In 2 dimensional electrophoresis, a spot with low molecular weigth and high pI is noted (arrow) and also several other spots are noted but not so prominent. pI marker proteins in the rod gel above the slab gel are cytochrome C2 (pI 6.2), myoglobin (pI 7.8–8.2), cytochrome C (pI 10.6). The molecular weight marker proteins are on the right of slab gel.

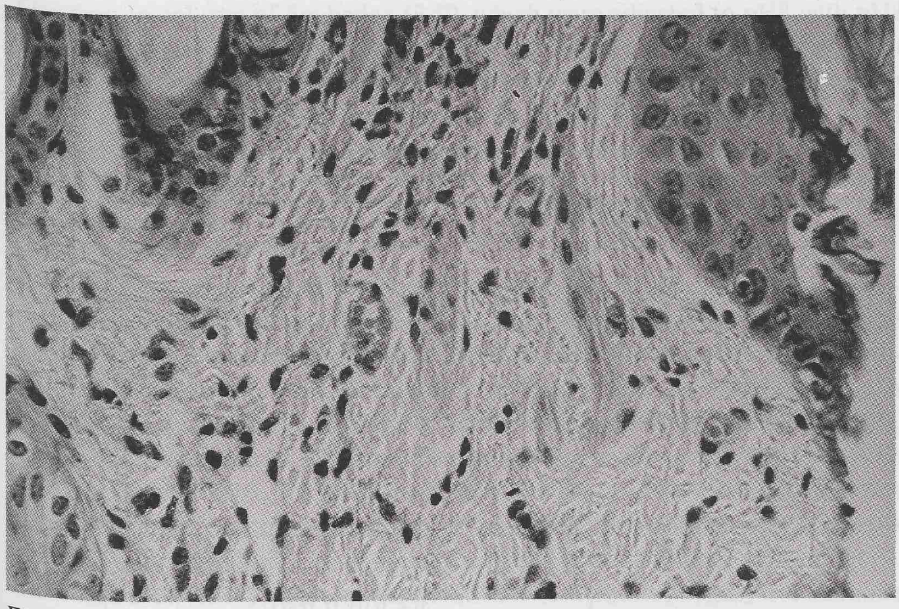

Figure 5. Mononuclear cells and few eosinophils in the dermis of guinea-pig skin injected with Ext A. H-E stain (250 x).

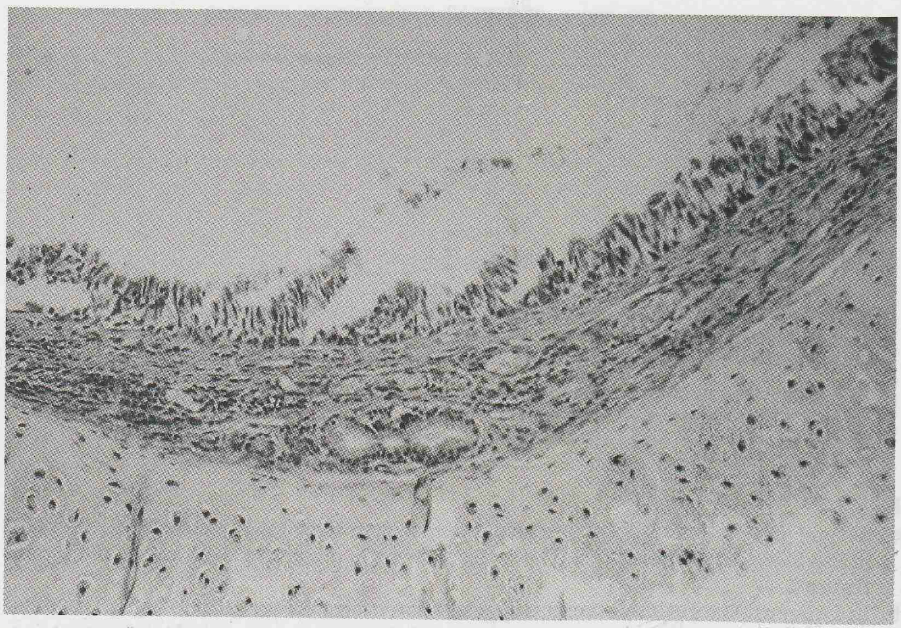

Figure 6. Tracheal rings of guinea-pig incubated with Ext A. Mild exfoliation and irregular lining are noted. H-E stain (250 x).

Table 1. The human nasal cilia activity tested by control solution and Ext A.

	PBS (PH 7.2)	0.1 mg/ml	1 mg/ml
Non-allergy N=5	270	180	80
	290	160	130
	250	160	130
	240	130	100
	210	150	110
Nasal allergy N=6	150	90	90
	140	80	70
	150	70	50
	180	120	120
	140	90	90

$p < 0.01$ $p > 0.05$

$p < 0.01$ (t test)

Unit: minute

Table 2. The human nasal cilia activity tested by control solution and fractions Ext A.

	Acetate-Tris HCl PH 8	Peak I 0.1 mg/ml	Peak II 0.1 mg/ml
Non-allergy	480	260	320
N=5	400	240	180
	360	160	140
	350	190	180
	220	160	120
Nasal allergy	280	220	140
N=6	270	120	150
	250	140	160
	240	150	120
	190	140	100
	180	160	100
		$p < 0.01$	$p > 0.05$
		$p < 0.01$ (t test)	

Unit: minute

b. In human nasal cilia beating with the fractions of the 1st and 2nd peaks by gel filtration on column of Sephadex G-50, which were adjusted to pH8 with pH8 Tris-HCl and concentrated and adjusted to 0.1 mg/ml as described by Gleich et al. (1974). There was an inhibitory effect of both high and low molecular weight proteins on the cilia activity compared with the pH8 acetate-Tris HCl buffer solution ($p < 0.01$) and no difference between the two fractions of proteins ($p > 0.05$) (Table 2).

DISCUSSION

The experimental results indicate that Ext B from biologically stimulated eosinophils contained MBP as well as Ext A' from physically destroyed eosinophils, and injured the nasal mucosal cilia. There, however, are a few problems to be discussed before conclusion. First, eosinophils possibly release Ext B biologically in incubation with IgG immune complex, because eosinophils, after the incubation, showed degranulation with normal cell membrane and cytoplasm, no destruction of cells and were viable in dye exclusion test. No release of LDH from eosinophils after incubation with immune complex suggests that specific granule contents such as peroxidase, MBP and so on were released without damage of eosinophils. Takenaka et al. (1977) have also described that eosinophils phagocytose immune complexes of IgG and IgE classes in the experiment similarly designed as the present one, and release peroxidase in immunoglobulin class specific manner

(releasable in IgG and IgE immune complexes but not in IgA and IgM). As a further problem, however, it should be confirmed that this release is an energy dependent event.

Secondary, MBP is possibly contained in Ext B as well as Ext A and A'. MBP constitutes no less than 50% of the total protein content of eosinophil granules with molecular weight about 9,300–12,600 daltons and high pH above 10 (Ackerman et al., 1983). In SDS electrophoresis, Ext A' and B showed a band of approximately 12K in molecular weight, about 10 in pH in isoelectric focusing, and belonged to the 2nd peak in gel filtration with Sephadex G-50. These results fit well with Gleich's reports.

Thirdly, even if Ext A and A' injured the nasal cilia, MBP contained in extracts is not the only chemical substance to contribute it. Because in the present study, extract of the 1st peak as well as the 2nd peak (predominantly MBP) inhibited cilia beating to a similar degree. The 1st peak contained two major and two minor bands in electrophoresis, and was rich in peroxidase. Eosinophil peroxidase, a major component of granule matrix, is found to be involved in cytotoxic processes against lung cells and to activate mast cell degranulation (Henderson et al., 1980; Davis et al., 1984; Agosti et al., 1985). In addition, it seems that eosinophil cation protein (21K) was contained in the 1st peak and took a cytotoxic function.

Fourthly, it is not yet confirmed that the substance released in the human nasal mucosa by allergic reaction is just the same as components in Ext B. Okuda et al. (1983) have documented that incubation of eosinophils with immune complex induces morphological degranulation as seen in allergic nasal mucosa and release granule content. We, however, need further study to clarify these questions.

In spite of the above discussions, Ext A, A' (1st and 2nd peaks) have injurious effect on nasal mucosa as well as tracheal epithelium. In the present study, Ext A and A' inhibited nasal cilia beating and induced exfoliation of tracheal epithelial cells of the guinea-pig. This confirms the Frigas reports (1980, 1981) that the incubation of bronchial epithelium in MBP induces the changes similar to asthma such as exfoliation of bronchial epithelial cells, loss of ciliated cells and cell disruption. On the other hand, the effect of Ext A was very slight on nasal provocation and skin reactions. Provocation with Ext A could not induce any nasal symptoms and intracutaneous injection in the guinea-pig induced only slight infiltration of mononuclear cells with a few eosinophils.

From the above discussions, it is likely that eosinophils appear in the allergic nasal mucosa, release granule contents and injure the nasal epithelium but don't contribute to nasal manifestation of symptoms. Of the granule contents released, peroxidase, eosinophil cation protein, eosinophil-derived neurotoxin etc. other than MBP may also have cytotoxic function.

CONCLUSION

1. MBP, peroxidase etc. are released from eosinophils by a biological mechanism.
2. The eosinophil extract injures nasal cilia activity as well as the tracheal mucosa of the guinea-pig, but has no effect on the manifestation of nasal allergic symptoms, or irritation to the skin of the guinea-pig.
3. Granule components other than MBP (mainly peroxidase) also contribute to the injurious effect on airway mucosa.

ZUSAMMENFASSUNG

Eosinophil verbindet sehr oft mit auftretender Nasenallergie in der Nasenschleimhaut und Nasensekretion.

Die Rolle der Eosinophil in der überempfindlichen Krankheit ist allerdings noch unbekannt, ob es das Gewebe schutzt oder zerstört und ob es Nährungszelle aktiviert.

Wirkzamkeiten der zwei Methoden von menschlichen Eosinophil-Extrakt (biologischer und körperlicher Extrakt) auf die Nasenschleimhaut werden durch Auftragen in dem Nasenreiztest und Zilien-Schlagtest und auch im Trachea-Ring-Inkubation und Hauttest beim Versuchskaninchen gemessen.

Ergebnisse der Forschung schlägt vor, dass das Hauptprotein von Eosinophil und andere Proteinbestandteile die Funktionen der Nasenschleimhaut und Trachea-schleimhaut des Versuchskaninchens zu beschädigen verursachen könnten.

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