

Eosinophilic apoptosis in sinus mucosa: Relationship to tissue eosinophilia and its resolution in allergic sinusitis

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Background: Apoptosis, which is regulated by both cell survival and death signals, is important for the swift clearance of unwanted cells.

Objective: We sought to elucidate whether eosinophilic apoptosis is associated with tissue eosinophilia and to determine its resolution in allergic sinusitis (AS).

Methods: Numbers of eosinophils, numbers of IL-5⁺ cells, and the apoptosis index of eosinophils were calculated in the submucosa (both superficial and deep layers) of patients with AS by using histochemical methods before and after prednisolone treatment. Patients without AS were used for control groups. Anti-EG2 antibody was used to identify eosinophils. IL-5, Fas, or Bax expression of eosinophils was evaluated to elucidate the role of the factors affecting eosinophilic apoptosis.

Results: EG2 and IL-5⁺ cells were abundant in the submucosa of patients with AS, especially in the superficial layer. About 50% to 60% of the IL-5-producing cells were eosinophils.

Apoptotic eosinophils were less numerous in the superficial layer than the deep layer in these diseases. After prednisolone treatment, an induction of eosinophilic apoptosis was accompanied by a significant decrease in the number of EG2⁺ and IL-5⁺ cells. No remarkable difference was observed in the Fas or Bax expression of eosinophils after prednisolone treatment.

Conclusion: Autocrine secretion of IL-5 from eosinophils may be one reason why eosinophilic disease is difficult to manage. Induction of eosinophilic apoptosis is critical for reversing tissue eosinophilia in patients with AS. (*J Allergy Clin Immunol* 2000;106:551-8.)

Key words: Eosinophil, apoptosis, IL-5, allergic sinusitis, sinus mucosa, EG2, Fas, Bax

In allergic sinusitis (AS) eosinophilic infiltration and persistence of eosinophils is the most outstanding feature of nasal mucosa.^{1,2} With the ability to generate potent toxic products (including cationic proteins, oxygen-free

Abbreviations used

AI:	Apoptotic index
APAAP:	Alkaline phosphatase antialkaline phosphatase
AS:	Allergic sinusitis
DAB:	3-3'-Diaminobenzidine tetrahydrochloride
HPF:	High-power field
NAS:	Nonallergic sinusitis
TUNEL:	Terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling

radicals, and proinflammatory cytokines), eosinophils play a major role in mediating injury to respiratory tissue undergoing the inflammation responsible for AS, as well as nasal polyposis, asthma, and aspirin-sensitive disorders.¹⁻⁵

The infiltration by eosinophils initially depends on an influx from systemic blood into local tissue. This influx is regulated by the chemokines RANTES, monocyte chemoattractant protein 3, macrophage inflammatory protein 1, and eotaxin and the inflammatory cytokines IL-3, IL-5, and GM-CSF. The process also requires the expression of celladhesion molecules. Hamilos et al⁶ reported that patients with allergic nasal polyposis had significantly higher tissue densities of IL-4, IL-5, GM-CSF, and IL-3 compared with those with nonallergic nasal polyposis. However, the extent to which tissue eosinophils accumulate is not only dependent on the number of migrated eosinophils but also on the duration of eosinophil survival in tissues. A persistent eosinophilic presence in a tissue will prolong the course of inflammation. Therefore the mechanism or mechanisms behind the survival and swift clearance of eosinophils are of great interest.

Apoptosis is a form of programmed cell death distinct from necrosis. Although the latter results in the loss of cell membrane integrity and the release of the cell's content, thus promoting inflammation, apoptosis involves the silent clearance of inflammatory cells without disgorgement of their histotoxic contents.^{7,8} In vitro studies have shown that eosinophils can undergo apoptosis and be subsequently phagocytosed as intact cells by macrophages without eliciting a proinflammatory reaction.⁹ The inhibition of this process is related to persistent eosinophil inflammation during which cytokines enhancing eosinophil survival, especially IL-5, are upregulated.^{10,11} Bax protein is one of the impor-

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tant apoptosis-promoting members of the bcl-2 family.¹² Overexpression of Bax by gene technology enhanced the sensitivity of cancer cells to the antichemotherapeutic agent.¹³ Fas signal is one of the important signals for apoptosis and acts through binding of Fas ligand to Fas in the membranes of cells.¹⁴ Although it remains unclear whether Fas and Bax act through a common signaling mechanism, it is speculated that the levels of IL-5 and Fas or Bax expression are important for determining eosinophil survival and apoptosis.

Although previous investigations have mainly focused on in vitro models, a delay of eosinophilic apoptosis may also occur and perpetuate chronic inflammation in vivo in nasal eosinophilic tissue.¹¹ In addition, induction of eosinophilic apoptosis accelerates the removal of eosinophils with subsequent clinical benefits for asthmatic disease.¹⁵ It is thus worth exploring whether the induction of apoptosis leads to the resolution of eosinophilic inflammation in AS.

METHODS

Patients and tissue samples

The 29 patients involved in this study ranged in age from 17 to 69 years. There were 19 men and 10 women. All patients had ethmoid and maxillary sinusitis and had undergone routine diagnostic procedures at the Department of Otolaryngology, Osaka Medical College. Specific IgE for 9 allergens (house dust mites, the major 5 Japanese pollens, dogs, cats, and fungus) was measured by using the CAP-RAST test in all patients. The diagnosis of AS was confirmed by the elevation of total serum IgE and elevation of at least one specific IgE against the common allergens measured by the CAP-RAST test (score >2), as well as by evidence of allergic nasal symptoms and signs. The CAP-RAST test was conducted in the absence of antigen provocation. Patients with nonallergic sinusitis (NAS) were confirmed to have nonallergic rhinosinusitis by means of the CAP-RAST. They had chronic rhinosinusitis that was strictly diagnosed by endoscopic findings, paranasal sinus x-ray tomography, clinical history, and symptoms. Patients with NAS had more than 1 year of the following symptoms: chronic perennial nasal congestion, chronic purulent rhinorrhea, chronic postnasal drip, olfactory disturbance, and discomfort in the regions of the sinuses. They had endoscopic findings of nasal polyposis, purulent discharge, and obstruction in the middle meatus. The shadow or the thickness of the mucosa in the maxillary sinuses and ethmoid cells were found with x-ray tomography. As our standard therapy, patients with NAS were given a 14-member macrolide, and patients with AS were given systemic antihistamines. All patients were recruited if they had not had therapy at least 1 month before the study. When all patients with NAS and 8 patients with AS had undergone nasal endoscopic surgery, the mucosa in the ethmoid and maxillary sinus was obtained. Five of 13 patients with AS were treated by standard oral administration of prednisolone (20 mg/d) for 2 weeks before the operation. The biopsy for the mucosa was performed before the administration of prednisolone, and second samples were obtained at the time of surgery. The mucosa in the ethmoid and maxillary sinus were obtained by using the forceps during the operation and then fixed in formalin. Paraffin-embedded blocks were then cut into 4- μ m thick consecutive specimens and prepared for the detection of different factors. All sections were routinely stained with hematoxylin and eosin for pathologic diagnosis to exclude other diseases.

Antibodies and reagents

The following antibodies and reagents were used: polyclonal rabbit anti-human IL-5 antibody (dilution 1:50; Genzyme, Cambridge, Mass); monoclonal anti-human eosinophil cationic protein antibody (Clone EG2, dilution 1:500; Pharmacia, Uppsala, Sweden); rabbit anti-human fas polyclonal antibody (dilution 1:40; Calbiochem, Cambridge, Mass); polyclonal rabbit anti-human Bax antibody (dilution 1:1000; Pharmingen, San Diego, Calif); Dako LSAB kit (Dako, Carpinteria, Calif); Dako alkaline phosphatase antialkaline phosphatase (APAAP) kit; ApopTag in situ kit (Oncor, Gaithersburg, Md); proteinase K (Wako, Osaka, Japan); ammonium nickel sulfate hexahydrate (Nacalai, Kyoto, Japan); and 3-3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan).

Immunohistochemistry

The avidin-biotin complex method was applied for immunohistochemical analyses. In brief, endogenous peroxidase activity of sections was quenched by the incubation with 3% hydrogen peroxide solution overnight. All sections were pretreated with blocking serum and then incubated with the first antibody at 4°C overnight. After being rinsed with pH 7.4 PBS, sections were incubated with the biotinylated second antibody for 40 minutes and allowed to react with streptavidin-peroxidase reagents for 30 minutes. Color visualization was achieved by using DAB chromogen and counterstaining with methyl green or hematoxylin. A negative control was included by using mouse immunoglobulin of the same isotype or rabbit serum as a replacement for the primary antibody. An additional control experiment was performed in IL-5 immunohistochemical assay with absorbance of the anti-IL-5 antibody by using recombinant IL-5. Antigen retrieval was carried out by using a microwave for Bax and a pressure cooker for Fas.

Double staining with IL-5 and EG2 antibody

According to the immunohistochemical method described above, IL-5 protein was detected first and developed as a black color by adding 0.04% ammonium nickel sulfate hexahydrate to the DAB solution. After sections were washed, they were reinoculated with anti-EG2 antibody and finally developed to show red color by using the APAAP method. The APAAP method was performed according to the instructions included with the Dako APAAP kit. Methyl green was used as a counterstaining reagent. Cells containing both red and black color were considered to be IL-5-expressing eosinophils.

Terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling method

Apoptotic cells were detected by using the terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) methods with the ApopTag in situ kit. The labeling targets are the multitude of new 3'-OH DNA ends generated by DNA fragmentation and are typically localized in morphologically identifiable nuclei of apoptotic cells, as previously described.¹⁶ The assay was performed as recommended by the manufacturer. Slides were pretreated with 3% hydrogen peroxide. The apoptotic cells were visualized with DAB chromogen in 0.01% H₂O₂ solution and counterstained with methyl green. The positive control slide supplied by the manufacturer was used in each set of assays.

Detection of apoptotic eosinophils

To identify which eosinophils were undergoing apoptosis, a pilot double-labeling technique was conducted combining TUNEL and avidin-biotin complex methods for bicolored staining of the apoptotic eosinophils. Briefly, TUNEL was performed first. The nuclei of apoptotic cells were labeled black by adding 0.04% ammonium nickel sulfate hexahydrate to the DAB solution. After these sections

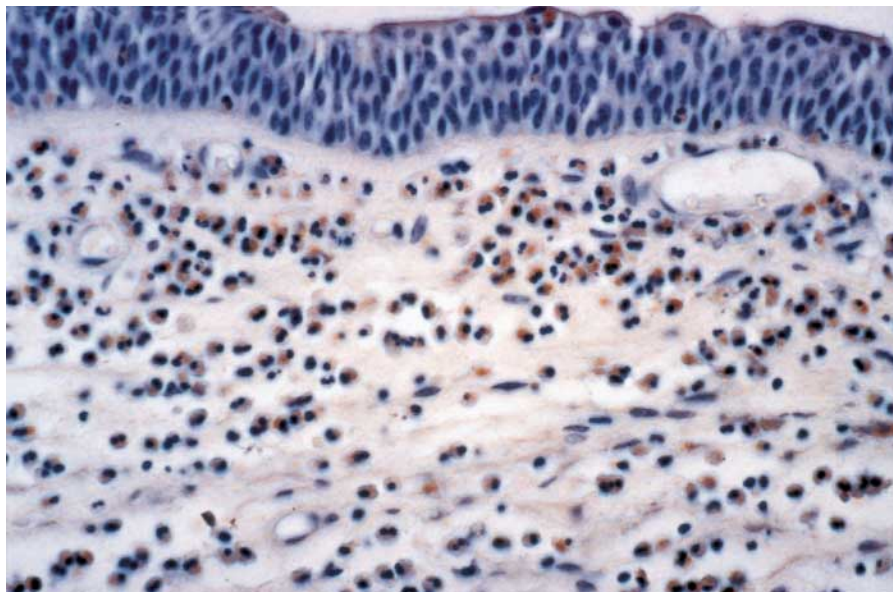


FIG 1. EG2⁺ cells accumulated in the mucosa of patients with AS, especially in the superficial layer (magnification 250 $\times$).

were washed with PBS, they were subjected to EG2 immunostaining. The cytoplasm of EG2⁺ cells was visualized as a brown color. Finally, the sections were counterstained with methyl green.

Apoptosis was also evaluated on the basis of the morphologic appearance. In particular, marked loss of cytoplasm and nuclear condensation are considered as the two major criteria. Apoptotic eosinophils were therefore identified as those cells stained by using the double-labeled technique and showing the morphologic appearance of apoptosis. This method of identification of apoptotic eosinophils was similar to that used by Vignola et al.¹⁷

Evaluation of slides

For microscopic examination, the lamina propria of mucosa was separated into a superficial and deep layer. The superficial layer was defined as the area just beneath the basement membrane (0–0.48 mm). The deep layer was the area 0.48 mm under the basement membrane (0.48–0.96 mm), which was decided by the scope diameter of the microscope under a magnification of 400 $\times$. All factors were evaluated in two layers under a magnification of 400 $\times$ by using a high-power field (HPF). For microscopic analysis, we selected 3 staining hot spots and counted positive cells. Each staining was scored independently by two doctors in a coded manner (without knowledge of the clinical parameters). We calculated the average of the 6 values to determine the cell number or the percentage of positive cells. The degree of eosinophil accumulation was expressed as EG2⁺ cells per HPF. IL-5 expression was expressed as IL-5⁺ cells per HPF. The apoptotic index (AI) of eosinophils was expressed as the apoptotic rate (apoptotic eosinophils per EG2⁺ cells). The percentage of IL-5⁺ eosinophils was expressed as the number of IL-5⁺ eosinophils per total number of IL-5⁺ cells. Fas⁺ or Bax⁺ eosinophils were recognized by morphologic features, which were characterized by a double lobule of nucleus and rich cytoplasm. The percentage of Fas⁺ or Bax⁺ eosinophils was determined as the number of Fas⁺ or Bax⁺ eosinophils per total number of eosinophils.

Statistical analysis

The SPSS 6.1 software package for Macintosh was used for all statistical analyses. The nonparametric Mann-Whitney test was

applied to compare the data among different diseases. The Wilcoxon matched-pairs rank test was used to analyze the difference of parameters between the superficial and deep layers in the same group.

RESULTS

Eosinophil accumulation in AS

To assess eosinophil accumulation, anti-EG2 antibodies, which recognizes eosinophils in tissue, were used (Fig 1). This anti-EG2 antibody bound specifically to eosinophil cationic protein secreted (or extracted) from eosinophils. Results are shown in Table I. The number of EG2⁺ cells was remarkably higher in the superficial and deep layer of the AS than the NAS mucosa ($P < .01$). The superficial layer was significantly richer in EG2⁺ cells than the deep layer within the AS ($P < .01$) and NAS groups ($P < .05$) by using the Wilcoxon matched-pairs rank test. The dominated infiltrating cells in allergic mucosa were EG2⁺ cells other than T cells, which were stained by anti-CD3 antibody. The percentages of eosinophil in the mucosa of patients with AS and that of patients with NAS were $85\% \pm 29\%$ and $63\% \pm 14\%$, respectively.

IL-5 overexpression in AS

The results of IL-5 immunohistochemical staining are given in Fig 2. The results of analysis of IL-5 expression are shown in Table II. IL-5⁺ cells were more abundant in the superficial ($P < .01$) and deep layers ($P < .01$) of allergic mucosa than the corresponding areas in patients with NAS. The IL-5⁺ cell number was higher in the superficial than in the deep area in tissue samples of AS ($P < .01$) and NAS mucosa ($P < .05$). The number of IL-5⁺ cells was much higher than the number of T cells, which were stained by anti-CD3 antibody (data not shown).

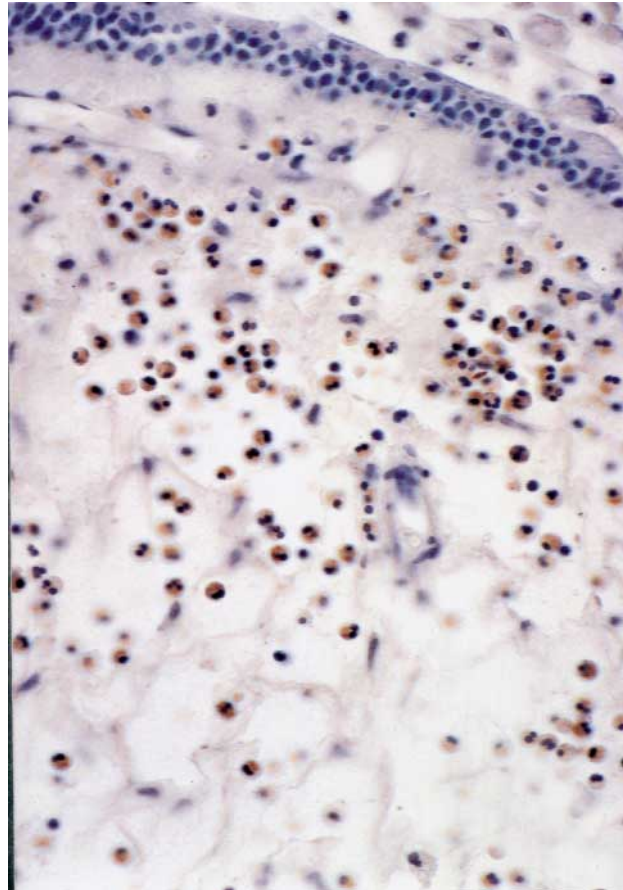


FIG 2. Immunohistochemical staining of IL-5-producing cells in allergic mucosa (magnification 250 $\times$).

TABLE I. Eosinophil EG2⁺ cells per HPF

Disease	Superficial, mean $\pm$ SD (median)	Deep, mean $\pm$ SD (median)	<i>P</i> value*
AS (n = 13)	114 $\pm$ 58 (102) [†]	47 $\pm$ 22 (51) [†]	<.01
NAS (n = 16)	15 $\pm$ 13 (18)	8 $\pm$ 9 (10)	<.01

*Comparison between superficial and deep layers in the same group by using the Wilcoxon matched-pairs rank test.

[†]*P* < .01 when compared with the same layer in patients with NAS by using the Mann-Whitney rank test.

Eosinophils expressing IL-5

Many IL-5⁺ cells (from 35.3%-77.1%) were identified by morphologic features as eosinophils. The mean and median percentages of eosinophils among IL-5⁺ cells are presented in Table III. No significant differences were found among the two groups or different areas. To detect which IL-5⁺ cells were eosinophils in allergic tissues, representative sections were double stained with anti-IL-5 and EG2 antibodies. The percentage of eosinophils among the IL-5⁺ cells was also high in double-stained sections, showing that a considerable fraction of IL-5⁺ cells were EG2⁺ cells (Fig 3). This phenomenon was confirmed when consecutive sections separately stained with EG2 and IL-5 antibodies were compared.

Expression of apoptotic eosinophils

The number and location of EG2⁺ or TUNEL⁺ cells detected separately in consecutive sections was consistent with the results obtained by double labeling. In double-labeled sections DNA fragments were detected in the nuclei of some EG2⁺ cells, indicating that these cells were apoptotic (Fig 4). There was faint nonspecific staining in necrotic cells and some eosinophils. Apoptotic cells could be distinguished by a simple morphologic examination.¹⁸ Table IV lists the values of the eosinophilic AI in the two groups of patients. The AI of eosinophil was higher in patients with AS (*P* < .01) than in counterparts with NAS. The index was lower in the superficial layer than in the deep layer in both the AS (*P* < .01) and NAS (*P* < .05) groups.

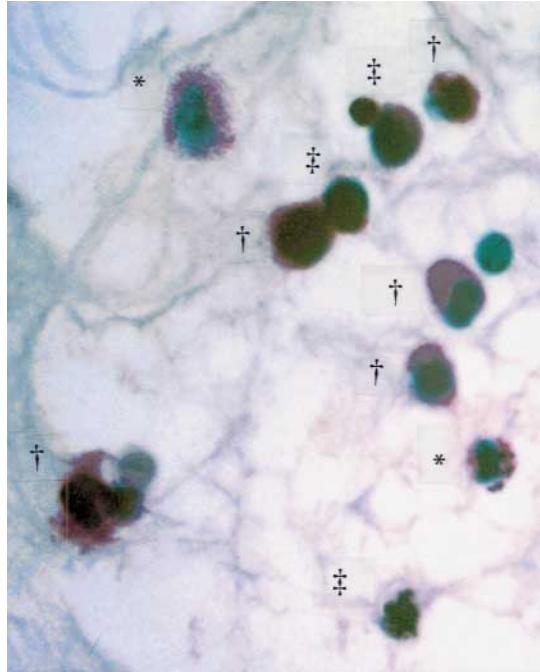


FIG 3. Double staining of IL-5-producing eosinophils. The mixture of red and black shows that the majority of IL-5-producing cells (black) were EG2⁺ (red) cells. Nuclei were counterstained with methyl green (magnification 600×). *EG2⁺/IL-5⁻ cells; †EG2⁺/IL-5⁺ cells; ‡: EG2⁻/IL-5⁺ cells.

TABLE II. Expression of IL-5⁺ cells per HPF

Disease	Superficial, mean $\pm$ SD (median)	Deep, mean $\pm$ SD (median)	P value*
AS (n = 13)	112 $\pm$ 47 (110) [†]	51 $\pm$ 27 (42) [†]	<.01
NAS (n = 16)	8 $\pm$ 11 (12)	4 $\pm$ 4 (5)	<.05

*Comparison between the superficial and deep layers in the same group by using the Wilcoxon matched-pairs rank test.

[†]P < .01 when compared with the same layer in patients with NAS by using the Mann-Whitney rank test.

TABLE III. Percentage of eosinophils among IL-5⁺ cells

Disease	Superficial, mean $\pm$ SD (median)	Deep, mean $\pm$ SD (median)	P value*
AS (n = 13)	58.1% $\pm$ 8.2% (59.3%)	54.9% $\pm$ 6.1% (55.7%)	NS
NAS (n = 16)	55.8% $\pm$ 9.5% (55.8%)	51.1% $\pm$ 9.3% (50.0%)	NS

No significance was found in the same layer among the two groups by using the Mann-Whitney rank test.

NS, Not significant.

*Comparison between the superficial and deep layers in the same group by using the Wilcoxon matched-pairs rank test.

Fas and Bax expression in eosinophils

To clarify whether Fas and Bax proteins play roles in the reduced apoptosis of eosinophils in AS, we searched for these two proteins in allergic mucosa. Eosinophils were identified by morphologic features. Some 81.1% to 97.9% of eosinophils expressed the Bax antigen. No noticeable difference was found in the two layers ($P = .40$; 93.6% $\pm$ 3.6% [median, 92.8%] in the superficial vs 92.5% $\pm$ 4.8% [median, 93.1%] in the deep layer). Also, many eosinophils expressed Fas (from 34.3%-69.3%). No significant difference of Fas expression in eosinophils was found in the two layers (52.4% $\pm$ 8.7% [median,

50.9%] in the superficial vs 48.0% $\pm$ 7.0% [median, 48.0%] in the deep layer). The expression rates of the two proteins in eosinophils did not differ significantly before and after steroid treatment (data not shown).

Effects of prednisolone therapy

Table V shows the effect of steroid therapy in 5 patients with AS. After prednisolone therapy, the number of EG2⁺ and IL-5⁺ cells was markedly decreased in 4 of 5 patients with an increased eosinophil AI. No inductive effect on eosinophilic apoptosis was observed in the remaining patient. As expected, the number of EG2⁺ and

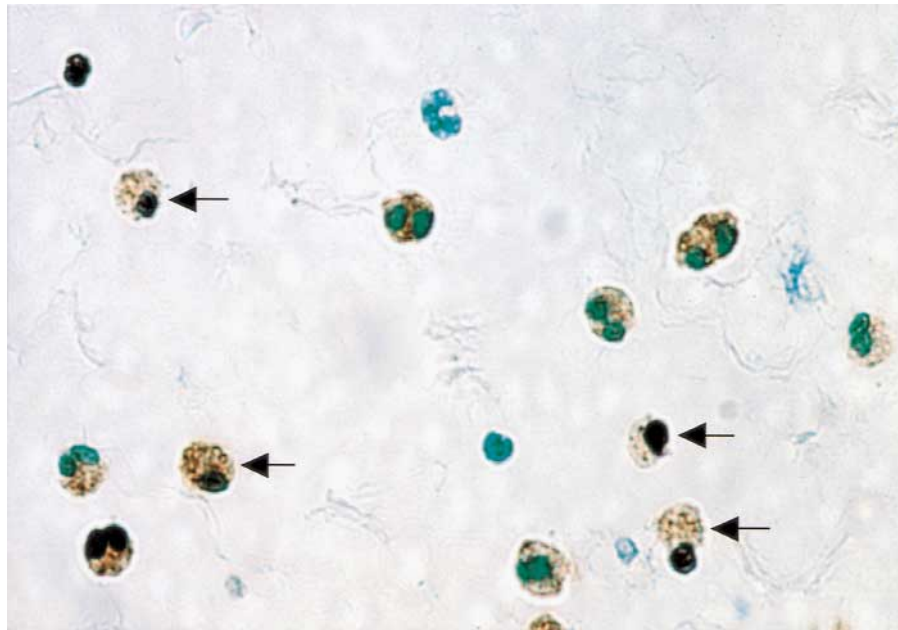


FIG 4. Apoptotic eosinophils (arrows). The apoptotic nucleus was labeled black by using the TUNEL method, whereas eosinophils appear as brown on immunohistochemical staining. Methyl green was used as a counterstaining reagent (magnification 600 $\times$).

TABLE IV. Apoptotic index of eosinophils

Disease	Superficial, mean $\pm$ SD (median)	Deep, mean $\pm$ SD (median)	P value*
AS (n = 13)	0.98% $\pm$ 0.86% (1.12%) [†]	3.99% $\pm$ 2.94% (3.08%) [†]	<.01
NAS	0.64% $\pm$ 1.13% (0.77%)	2.24% $\pm$ 3.06% (2.57%)	<.05

*Comparison between the superficial and deep layers in the same group by using the Wilcoxon matched-pairs rank test.

[†]P < .01 when compared with the same layer in patients with NAS by using the Mann-Whitney rank test.

IL-5⁺ cells was not reduced in this patient. Additionally, the percentage of IL-5⁺ eosinophils changed only slightly in the 5 patients after therapy (56.6% $\pm$ 5.1% vs 57.2% $\pm$ 11.1% in the superficial layer and 53.1% $\pm$ 7.8% vs 54.2% $\pm$ 8.5% in the deep layer).

DISCUSSION

In this study we revealed that EG2⁺ cells were abundant in allergic mucosa and dominated the superficial area, pointing to eosinophils as the main effector mediating inflammation in allergic tissue. To explore whether this accumulation was associated with eosinophilic apoptosis, we examined the eosinophil AI in superficial and deep layers of the mucosa. The low AI in the superficial layer was consistent with the theory that inhibition of apoptosis contributes to eosinophil accumulation.^{10,11} Recently, several reports have shown that eosinophilic apoptosis was modulated by cytokines that enhance eosinophil survival.^{10,11,19,20} IL-5 is the most powerful of these cytokines.^{10,11,19} Our finding that the superficial layer rich in IL-5⁺ cells had a lower eosinophilic AI than the deep layer supports this hypothesis. Previous reports

have suggested that the upregulation of IL-5 was caused by secretion by T cells. Because lymphocytes do not store cytokines, immunohistochemistry largely underestimates T_H2 cytokine-positive cells. However, in these samples of allergic tissue, the number of IL-5⁺ cells was much higher than the number of T cells, and the number of T cells did not differ between AS and NAS mucosa. We conclude that the difference of the proportion of IL-5-producing cells between AS and NAS mucosa was due to the difference of the number of EG2⁺ cells, suggesting that IL-5 production by eosinophils may contribute to the pathogenesis of AS. It is also unlikely that mast cells contribute much to the IL-5 level because the number of mast cells did not significantly differ between allergic and normal subjects.²¹ In accordance with the opinion of Wedi et al,²² we concluded that an autocrine and paracrine use of IL-5 provides pathways to maintain migration, viability, and effector functions of these cells. Other survival-enhancing cytokines, such as IL-3 and GM-CSF, also can be synthesized by eosinophils themselves. Therefore the induction of eosinophil apoptosis is critical to reverse tissue eosinophilia in allergic sinusitis.

Bearing the above background in mind, the finding

TABLE V. Effect of steroid treatment on allergic sinusitis.

Patient No.	Superficial			Deep		
	AI	EG2	IL-5	AI	EG2	IL-5
1						
Before	0.55%	122	146	1.62%	54	68
After	2.36%	49	70	5.51%	38	46
2						
Before	0.62%	106	109	2.02%	33	38
After	1.52%	83	90	4.54%	22	24
3						
Before	0.60%	265	270	0.86%	161	169
After	1.40%	146	151	3.50%	114	121
4						
Before	1.00%	99	109	0.00%	66	73
After	6.00%	44	53	4.50%	41	48
5						
Before	0.00%	21	19	1.80%	18	17
After	0.00%	31	33	0.00%	24	28

Data for each factor (AI, EG2, and IL-5) are expressed as the average number of positive cells per HPF.

that EG2⁺ eosinophils and apoptotic eosinophils are both more prevalent in AS mucosa than in NAS mucosa should be interpreted with caution.

First of all, unlike in the *in vitro* experiments, tissue eosinophil accumulation *in vivo* was regulated not only by cell death but also by the eosinophil influx from the circulation into tissues. Recently, several eosinophil chemotactic molecules have been identified. Cooperation between the eosinophil chemotactic factors and the eosinophil survival factor IL-5 can markedly enhance the accumulation of eosinophils induced by either of these factors alone.^{23,24} Indeed, it has been shown that the expression of eotaxin, an eosinophil-specific chemotactic factor, was upregulated in allergic epithelium compared with that of normal control subjects.²⁵ We also detected eotaxin expression in the AS epithelium (data not shown). Therefore an influx of eosinophils induced by chemotactic factors may be the basis for the disease in our subjects, whereas delayed apoptosis serves to augment this effect.

Second, eosinophils in allergic tissue will still undergo apoptosis over the course of the disease. We demonstrated that a large population of eosinophils expressed the Fas and Bax protein in allergic mucosa, and the rate at which these apoptosis-driving proteins were expressed did not differ between the superficial and deep layers. Other researchers have reported similar results that Fas receptor was expressed in allergic eosinophils at levels not different from those in eosinophils isolated from non-allergic subjects.²² We speculate that delayed eosinophilic apoptosis in allergic disease is more dependent on the level of survival signals than the abrogation of the cell death pathway. The apoptotic pathway for eosinophils can be activated in an allergic mouse model or in cultures containing IL-5.^{26,27} When fresh eosinophils purified from the peripheral blood of atopic individuals were cultured in medium containing IL-5, the proportion of apoptotic eosinophils progressively

increased with time, although this increase was delayed compared with those of cells not treated with IL-5.¹⁷ On the other hand, it has been suggested that cells undergoing apoptosis may lose their characteristic granules, and therefore the sequential TUNEL and EG2 staining may underestimate the numbers of eosinophils undergoing apoptosis.²⁸ However, we do not think that such an underestimation accounts for the significant difference of eosinophilic AI in between superficial and deep layers or between AS and NAS groups.

The degree of tissue eosinophilia has been suggested to be associated with the severity of allergic disease. Depletion of eosinophils in tissues improves the clinical symptoms and signs. However, the decrease of eosinophil influx can only partly explain the reduction in tissue eosinophil number. It cannot explain how eosinophils already present in local tissue are removed. Methods accelerating apoptosis of eosinophils could be helpful for eosinophil depletion in AS and successful treatment of this disease. Some drugs have been shown to counteract the prolonged eosinophil survival by inducing eosinophil apoptosis.^{12,29,30} Glucocorticoids are widely recognized as antiallergic and antihypereosinophilic agents. In this study we administered prednisolone to 5 patients with AS. After this treatment, the number of eosinophils and IL-5⁺ cells was reduced, with an increase of eosinophilic apoptosis in 4 of 5 patients. However, the percentage of eosinophils expressing IL-5 did not change significantly. Because of autoproduct of IL-5 in eosinophils, the direct induction of eosinophilic apoptosis may be one reason for the decrease of IL-5⁺ cells and further resolution of tissue eosinophilia. This hypothesis is supported by the observation that steroids can induce eosinophil apoptosis through the glucocorticoid receptor on the surface of the eosinophils.³¹ This is well in accord with the clinical observation that glucocorticoids have a quick and marked effect on allergic diseases, even if administered after the early-phase reaction. More impor-

tantly, apoptotic eosinophils, unlike necrotic cells, could be swiftly removed by macrophages without eliciting preinflammatory reactions.⁹ Thus the induction of eosinophilic apoptosis is central to prevention of the presence of eosinophils in allergic mucosa. Unfortunately, along with a lack of response to prednisolone seen in one patient in this study, corticosteroid resistance has posed a therapeutic problem.³² There is no simple model for the treatment of all allergic diseases.

The final event is the removal of the apoptotic eosinophils. Macrophage phagocytosis of apoptosis cells accounts for this clearance, which prevents the release of proinflammatory contents.^{9,20,33,34} However, this event can be inhibited by various microenvironmental factors, including the low pH, accumulation of cationic molecules, or high concentrations of fibronectin or vitronectin, which may be present in eosinophilia tissue. This may result in the delayed removal and secondary necrosis of apoptotic eosinophils. An *in vitro* study^{9,34} revealed that if these postapoptotic remnants are ingested by macrophages, they elicit secretion of proinflammatory mediators. Thus further investigation of whether the removal of apoptotic eosinophils is accelerated after steroid administration should be performed.

Taking all the observations together, we propose the acceleration of eosinophilic apoptosis as a therapeutic strategy for eosinophilic inflammation in nasal allergic disease. Identification of effective drugs will be a promising research field for this purpose.

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