

Immunohistology of the nasal mucosa in seasonal allergic rhinitis: Increases in activated eosinophils and epithelial mast cells

Andrew M. Bentley, MD,* ** Mikila R. Jacobson, PhD,**

Vincent Cumberworth, MD,* Julia R. Barkans,** Redwan Moqbel, PhD,**

Lawrence B. Schwartz, MD, PhD,*** Anne-Marie A. Irani, MD,***

A. Barry Kay, MD, PhD,** and Stephen R. Durham, MD* ** London, England, and Richmond, Va.

The immunohistology of the nasal mucosa was examined in 13 grass pollen-sensitive patients and in seven normal nonatopic control subjects before and during the pollen season. Cryostat sections (6 μ m) of biopsy specimens from the inferior turbinate were immunostained with the alkaline-phosphatase antialkaline-phosphatase method and a panel of monoclonal antibodies. Mast cell subtypes were measured with a double sequential immunostaining method. Within the submucosa, seasonal increases in total (MBP⁺, $p < 0.01$) and "activated" (EG2⁺, $p < 0.01$) eosinophils were observed for the patients, which were significant when these counts were compared with counts for those of control subjects (MBP⁺ $p < 0.01$; EG2⁺ $p < 0.001$). Within the nasal epithelium, seasonal increases in total ($p < 0.05$) and "activated" ($p < 0.02$) eosinophils were also observed. Mast cell counts revealed seasonal increases in tryptase-only positive mast cell (MC_T) ($p < 0.02$) but not chymase plus tryptase-positive mast cells (MC_{TC}) within the epithelium that were significant when counts were compared with those of control subjects ($p < 0.03$). No significant changes were observed within the submucosa or epithelium for total leukocytes (CD45⁺ cells) or T-lymphocytes (CD3⁺, CD4⁺, CD8⁺, and CD 25⁺ cells) for either group. Similarly, no significant changes were observed for neutrophils (antielastase), macrophages (CD68⁺), nor HLA-DR⁺ cells. In the subjects with rhinitis, seasonal submucosal CD3⁺ counts correlated with MBP⁺ eosinophils ($r = 0.56$; $p < 0.05$) and MC_Ts ($r = 0.65$; $p < 0.02$). Similarly, seasonal epithelial EG2⁺ eosinophil counts correlated with MC_Ts ($r = 0.56$; $p < 0.05$). The results indicate that seasonal allergic rhinitis is associated with local accumulation of "activated" eosinophils and the epithelial migration of MC_Ts but not MC_{TC}s during natural grass-pollen exposure. (J ALLERGY CLIN IMMUNOL 1992;89:877-83.)

Key words: Eosinophils, macrophages, mast cells, nasal biopsies, neutrophils, rhinitis, seasonal, T-lymphocytes

Seasonal grass pollen-induced AR is a common cause of morbidity. During the past 30 years, there has been a fourfold increase in general practitioner consultations for hay fever in the United Kingdom.¹

From the *Nose Clinic, Royal Brompton and National Heart and Lung Hospital, and **Department of Allergy and Clinical Immunology, National Heart & Lung Institute, London, England, and ***Medical College of Virginia, Virginia Commonwealth University, Richmond, Va.

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Reprint requests: S. R. Durham, MD, Department of Allergy and Clinical Immunology, National Heart & Lung Institute, Dovehouse St., London, SW3 6LY, England.

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Abbreviations used

ECP:	Eosinophil cationic protein
MBP:	Major basic protein
MC _T :	Tryptase-only positive mast cell
MC _{TC} :	Chymase plus tryptase positive mast cell
TBS:	Tris-buffered saline
IL:	Interleukin
MAb:	Monoclonal antibody
AR:	Allergic rhinitis
HPF:	High-power field

The mechanisms of allergen-induced rhinitis have been studied by use of measurements performed in nasal washings after local allergen provocation and during natural seasonal exposure. Characteristic fea-

tures include increases in local concentrations of mediators of hypersensitivity² and an increase in inflammatory cells in which eosinophils predominate.³ Recent studies involving biopsy of the nasal mucosa and conventional histologic staining methods have demonstrated degranulation of mast cells after experimental allergen provocation⁴ and an increase in epithelial mast cells during the pollen season.⁵ This seasonal increase in epithelial mast cells was inhibited by topical corticosteroids.⁶ One study has demonstrated an increase in epithelial CD1⁺ cells, presumed Langerhans cells, during seasonal grass-pollen exposure.⁷ We recently demonstrated that 24 hours after local nasal allergen provocation, there was an increase in CD4⁺ cells and CD25⁺ cells and local accumulation of eosinophils and neutrophils.⁸ No significant changes were observed in the MC_T and MC_{TC} subtypes. In the present study, we tested the hypothesis that natural seasonal exposure to grass pollen is associated with tissue eosinophilia and that this may occur as a consequence of the local accumulation of either T-lymphocytes or mast cells.

PATIENTS, MATERIAL, AND METHODS

Thirteen patients (six male and seven female patients; mean age, 28.5 years; range, 20 to 48 years) were selected on the basis of a history of symptoms of rhinitis confined to the May to July grass-pollen season, a positive skin prick test (wheal diameter, >5 mm) to timothy grass-pollen extract (*Phleum pratense*; ALK, Horsholm, Denmark), and a positive RAST to timothy-grass pollen. Three patients had a grade 4 RAST; 7, grade 3; and 3, grade 2 (Phadebas [Phadebas RAST, Pharmacia Diagnostics AB, Uppsala, Sweden] scale 0 to 4). Patients were excluded if they gave a history of perennial allergy, had previously received immunotherapy, were smokers, or had taken topical or oral medication in the previous 1 month. Seven normal healthy control subjects (five male and two female subjects; mean age, 28.7 years; range, 20 to 34 years) were also studied. Subjects had no history of allergies, were nonsmokers, and had negative skin prick tests to a panel of common aeroallergens (timothy-grass pollen, tree pollen, weeds and shrubs, house dust, house dust mite, cat dander, dog hair, horse hair, feathers, *Aspergillus*, *Alternaria*, and *Cladosporium*).

Study design

The study was performed with the approval of the Royal Brompton National Heart and Lung Hospital Ethics Committee and with the patients' written informed consent. The patients attended on two occasions. Preseasonal biopsies were performed in January to April 1990 at a time when patients were asymptomatic. The side of the nose, on which the first biopsy was performed, was randomly selected in advance by computer-generated random numbers. In eight of the 20 subjects, out-of-season biopsies were performed in January/February 1991. All patients and control subjects

completed diary cards of their symptoms and rescue medication from May through July 1990. Nasal symptoms were recorded as sneezing, nasal blocking, and discharge, each on a severity scale of 0 to 3. The mean cumulative score of these symptoms was expressed weekly. Patients also recorded their daily use of antihistamine capsules (acrivastine, 8 mg). Medication scores were expressed as a weekly total of capsules taken. All subjects attended for a second nasal biopsy on the opposite side in the first week of July when the patients were symptomatic and grass-pollen counts were at their peak.

Nasal biopsy

Local anesthesia was achieved by applying a small cotton wool plug soaked in 3% cocaine and 0.025% adrenaline immediately below the inferior turbinate for 10 minutes. A 2.5 mm biopsy specimen was obtained with Gerritsma forceps.⁹ Any slight local bleeding was controlled by cautery with a silver nitrate stick. Patients were observed for 1 hour and then discharged.

Immunohistology

Nasal biopsy specimens were immediately placed in phosphate-buffered saline and within 15 minutes were transferred to a drop of ornithine carbamyl transferase-embedding medium (BDH, Merck, Ltd., Dagenham, Essex, England) on a small piece of card. The biopsy specimens were snap frozen in isopentane precooled in liquid nitrogen and stored at -80° C for a maximum period of 1 month. Six micron cryostat sections were cut, air dried for 1 hour, and then fixed by immersion in a mixture of acetone/methanol (60:40) for 7 minutes. After an additional 1 hour drying step, slides were stored at -20° C until stained. Immunostaining was performed with the modified alkaline-phosphatase antialkaline-phosphatase (AAP) method previously described.^{10, 11} All biopsy specimens were cut and stained within 1 month, thereby avoiding any "storage" effect. The same batch of MAbs was used throughout. The MAbs used were CD45 (HLe-1), CD3 (Leu-4), CD4 (Leu-3A), CD25 (anti-IL-2 receptor) (Becton Dickinson, Ltd., Cowley, Oxford, U.K.), CD8, HLA-DR, neutrophil elastase, CD68 (DAKO Ltd., High Wycombe, Buckinghamshire, U.K.), and EG2, which recognizes the cleaved secreted form of ECP representing "activated" eosinophils.^{12, 13} (Bradsure Biologicals, Loughborough, Leicestershire, U.K.). Anti-MBP antibody (BMK13) that binds to the 13.8 kd eosinophil granule MBP, was used as a pan-eosinophil marker, since it stains both resting and activated eosinophils.¹⁴ Human tonsil (obtained at routine tonsillectomy) and mouse IgG1 and IgG2 myeloma proteins were used as positive and negative controls in each staining run. Immunostaining of biopsy specimens from allergen-induced late cutaneous responses were used as a positive control for eosinophils (anti-MBP and EG2) and neutrophils (anti-elastase).

Mast cell subtypes were examined with a sequential double immunostaining method with MAbs B7 (antichymase) conjugated to biotin and G3 (antitryptase) conjugated to alkaline-phosphatase.¹⁵ Sections were fixed in Carnoy's fluid

(60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 15 minutes. Endogenous tissue peroxidase was inhibited by preincubating sections with 0.6% hydrogen peroxide in methanol for 30 minutes at room temperature. After a wash in TBS with 0.05% Tween, pH 7.4, sections were incubated with B7 (2.6 $\mu\text{g}/\text{ml}$ in TBS/20% human serum) overnight. After additional washings, sections were incubated with a 1:50 dilution of peroxidase-conjugated streptavidin in TBS containing 20% normal human serum for 1 hour and developed with 3,3 diaminobenzidine tetrachloride for 8 minutes (MC_{TS} stained brown). After an additional wash, sections were incubated with G3 antibody (6 $\mu\text{g}/\text{ml}$ in TBS/20% human serum) overnight and developed with fast-red salt (1 mg/ml in 0.1 mol/L of Tris, pH 8.2, containing naphthol AS-MX phosphate, 0.2 mg/ml) and levamisole (0.1 mmol/L) for 20 minutes (MC_{TS} stained red; MC_{TS} retained the initial stain and appeared red/brown). Sections were counterstained with Gill's hematoxylin No. 1 for 20 seconds, washed in tap water, and mounted in Kaiser's mounting medium.

Quantitation

Slides were counted "blind" by a single observer in coded random order with an Olympus (Olympus Corp., Lake Success, N.Y.) microscope with an eyepiece graticule (0.202 mm^2) at original magnification $\times 200$. At least two sections and a median of eight fields were counted for each MAb. The graticule was orientated along the epithelial basement membrane, and subepithelial counts were expressed as mean counts per HPF. Additionally, epithelial cell counts were expressed per unit area (square millimeter) of epithelium, measured with a computerized graphics tablet. The mean intraobserver coefficient of variation for repeat counts of all MABs was $< 8\%$.

Statistical analysis

Nonparametric methods were used because most cells within the mucosa revealed a positive skewed distribution. For individual subjects, cell counts before and during the pollen season were analyzed with the Wilcoxon's matched-pairs signed-ranks test (intragroup analysis). The changes in cell counts (before to peak season) in the patients compared with that of control subjects were analyzed with the Mann-Whitney U test (intergroup analysis).

RESULTS

Grass-pollen counts during the 1990 pollen season, nasal symptom scores, and use of antihistamines for the patients and control subjects are recorded in Fig. 1. Patients developed mild to moderate symptoms during a pollen season that was average, but short, for the United Kingdom. Increases in symptoms and antihistamine use paralleled the changes in the pollen count (Fig. 1). Median cell counts infiltrating the nasal submucosa and epithelium before and at peak season are represented in Table I. Counts for both patients and control subjects revealed a prominent leukocyte

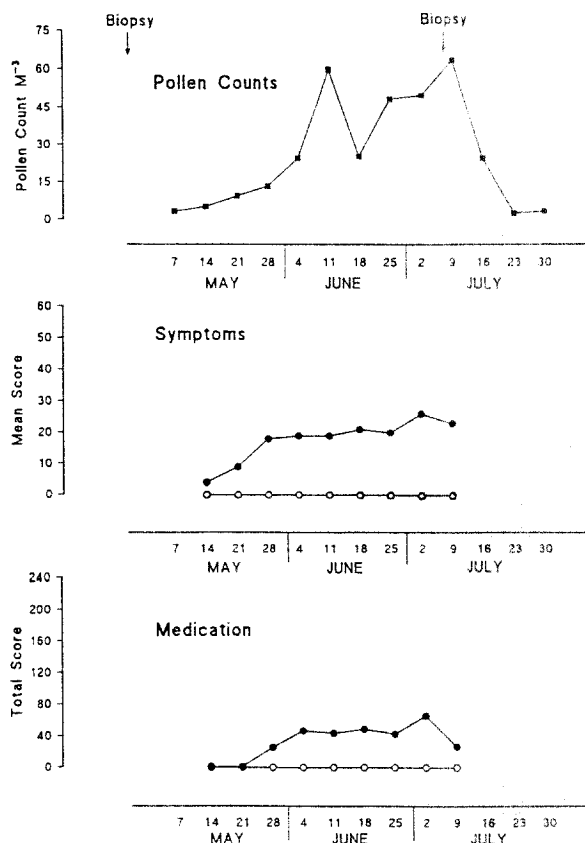


FIG. 1. Nasal symptom and medication scores in relation to weekly grass-pollen counts. Nasal symptoms are expressed as mean cumulative score of nasal blockage, sneezing, and discharge. Medication scores are expressed as total number of tablets (acrivastine, 8 mg) consumed per week; patients ($\bullet$); control subjects ($\circ$).

infiltrate within the submucosa. Positive staining for T-lymphocytes, eosinophils, neutrophils, and mast cells was also observed within the nasal epithelium. Patients with hay fever demonstrated a marked seasonal increase in total (MBP^+ , $p < 0.01$) and "activated" (EG2^+ , $p < 0.01$) eosinophils within the nasal submucosa. These increases were significant when they were compared with the corresponding changes for the control subjects (MBP^+ , $p < 0.01$; EG2^+ , $p < 0.001$) (Fig. 2). For the subjects with rhinitis, seasonal increases were also observed for MBP^+ ($p < 0.05$) and EG2^+ ($p < 0.02$) cells within the nasal epithelium. Mast cells were identified both in the submucosa and within the nasal epithelium. There were no seasonal changes in either MC_{TS} or MC_{TS} within the submucosa. In contrast, seven of the 13 patients demonstrated seasonal increases in MC_{TS} in the nasal epithelium ($p < 0.02$). These changes were significant when they were compared with changes in control subjects ($p < 0.03$) (Fig. 2).

TABLE I. Median cell counts per high-power field in the nasal submucosa and per square millimeter in the nasal epithelium

	Patients			Control subjects			p Values
	Preseason	Peak season	Median differences (95% CI)	Preseason	Peak season	Median differences (95% CI)	
Submucosa							
CD45	76.3	93.4	35.8 (−34.0,63.4)	98.0	97.7	27.5 (−34.6, 156.4)	NS
CD3	54.0	35.1	11.7 (−38.9, 30.0)	35.8	60.0	16.4 (−54.2, 145.7)	NS
CD4	38.0	42.9	16.4 (−1.4, 30.3)	30.4	47.2	29.2 (−9.0, 104.3)	NS
CD8	9.1	18.3	2.0 (−6.1, 22.2)	4.7	17.2	7.5 (−3.7, 32.4)	NS
IL-2R	0.8	0.4	−0.1 (−1.2, 0.8)	0.5	0.5	0.5 (−0.5, 2.1)	NS
EG2	1.8	6.5*	6.5 (0.7, 11.0)	0.6	0	−0.4 (−1.6, 0)	<0.001
MBP	0.4	8.8*	4.4 (0.6, 16.3)	0.8	0	−0.8 (−1.9, 0.4)	<0.01
CD68	15.3	16.7	2.2 (−10.0, 11.0)	16.9	21.3	7.8 (−5.1, 56.4)	NS
Elastase	4.6	4.8	−0.1 (−3.5, 10.2)	3.2	4.0	1.0 (−1.5, 15.4)	NS
HLA-DR	22.4	11.0	−1.1 (−16.6, 11.2)	6.5	20.7	4.2 (−9.8, 10.4)	NS
MC _T	1.9	2.3	0.2 (−1.5, 1.1)	3.3	1.8	−0.1 (−4.1, 2.3)	NS
MC _{TC}	5.0	4.1	1.4 (−3.8, 1.9)	5.1	5.3	−0.1 (−3.4, 2.4)	NS
Epithelium							
EG2	0	8†	8.0 (0, 48.8)	0	0	0 (0, 0)	<0.05
MBP	0	2‡	2.4 (0, 27.1)	0	0	0 (0, 1.8)	NS
MC _T	0	1.4†	1.4 (0, 15.7)	0	0	0 (−1.7, 5.4)	<0.03
MC _{TC}	0	0	0 (0, 2.3)	0	0	0 (−0.4, 0)	NS

CI, Confidence interval; NS, not significant.

The between-group analysis was performed with a Mann-Whitney U test.

The within-group analysis was performed with a Wilcoxon's matched-pairs, signed-rank test. Levels of significance:

* $p < 0.01$.

† $p < 0.02$.

‡ $p < 0.05$.

When total leukocyte counts (CD45⁺ cells) for the patients and control subjects were compared, no significant differences were observed either within or between the groups. Cell counts for T-lymphocytes and their subsets (CD3⁺, CD4⁺, CD8⁺, and CD25⁺) did not change either in the submucosa (Table I) or within the epithelium (data not presented). Similarly, no changes in the CD4⁺/CD8⁺ cell ratio were observed. There were no detectable changes in neutrophils (elastase), macrophages (CD68⁺), nor in positive immunostaining for HLA-DR.

In peak seasonal biopsy specimens from the subjects with rhinitis, significant correlations were observed between submucosal CD3⁺ cell counts and total eosinophils ($r = 0.56$; $p < 0.05$) and MC_Ts ($r = 0.65$; $p < 0.02$). A significant correlation was also observed between seasonal MC_Ts and EG2⁺ eosinophils within the nasal epithelium ($r = 0.56$; $p < 0.05$). Representative examples of the immunostaining patterns for eosinophils and mast cells are illustrated in Fig. 3.

DISCUSSION

We have demonstrated that during seasonal grass pollen-induced rhinitis, there is an increase in total

and actively secreting eosinophils within the nasal submucosa and epithelium. In addition, we have identified an increase in MC_Ts but not MC_{TC}s, which were confined to the epithelium. In contrast, no changes in either total leukocytes, lymphocytes, macrophages, or neutrophils were observed for either subjects with rhinitis or normal nonatopic control subjects.

There are both similarities and differences when the present changes after natural seasonal exposure are compared with our previous findings after local allergen provocation.⁸ This finding is perhaps not surprising when local provocation with allergen solution during a period of minutes is compared with that of natural exposure to particulate allergen during a prolonged period. We identified tissue eosinophilia in both circumstances, whereas the local increase in neutrophils that we observed after allergen provocation was not apparent after natural exposure. Conversely, during the pollen season, we observed an increase in epithelial mast cells, whereas we⁸ and other investigators¹⁶ have not detected significant changes in mast cell numbers after local allergen challenge. We did not identify an increase in CD4⁺ T-lymphocytes nor in CD25⁺ (activated IL-2R bearing) cells that we previously observed after local allergen chal-

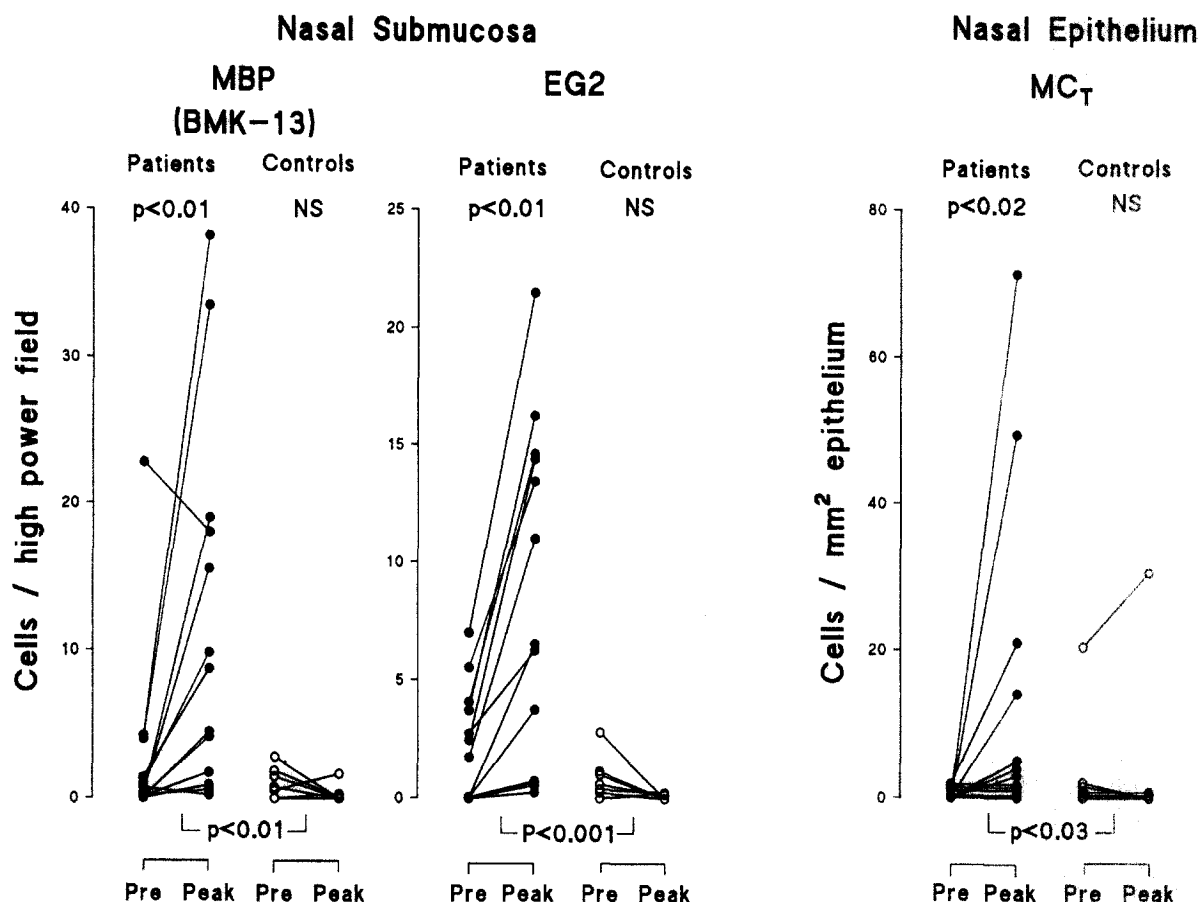


FIG. 2. Eosinophils in nasal submucosa and MC_Ts in nasal epithelium. Biopsies were performed before (*pre*) and midseason (*peak*) in both patients and control subjects. Total eosinophils were enumerated with BMK13 (anti-MBP), "activated" eosinophils with EG2, and MC_Ts were identified with G3 (antitryptase). Cell counts are expressed per HPF in submucosa and per square millimeter in epithelium; *P* values are presented; *NS*, not significant.

lenge. Thus, our findings during natural seasonal exposure suggest that tissue eosinophilia that is characteristic of AR may possibly occur as a consequence of the release of products derived from mast cells. The positive correlation between MC_Ts and "activated" eosinophils within the epithelium supports this association, although recruitment of mast cells and eosinophils in parallel cannot be excluded. Both T-lymphocytes¹⁷⁻²⁰ and mast cell lines^{21, 22} are known to produce a range of cytokines, including IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor that possess eosinophil-modifying properties. Clearly, the release of cytokines derived from T cells may not be apparent when T cell numbers alone are counted. A possible role for T cells is supported by the observed positive correlations between submucosal CD3⁺ cells and both eosinophils and MC_Ts during the season. Additional studies involving specific immunostaining for cytokines possibly combined with

in situ hybridization methods to detect mRNA for specific cytokines²³ will be required to determine whether local production of cytokines by T cells or mast cells may be involved in the local tissue eosinophilia in seasonal rhinitis.

In previous studies, degranulation of mast cells has been observed after experimental, immediate, allergen-induced rhinitis,²⁴ and nasal mast cell numbers were increased during the pollen season.⁵ A reduction in mast cell counts⁶ and a decrease in the histamine content of the nasal mucosa²⁵ have been observed after topical glucocorticoid treatment. In this study, the selective seasonal increase in MC_Ts, but not MC_{TC}s, that we observed was confined to the nasal epithelium. This finding may result from redistribution of mast cells during the pollen season with migration into the epithelium. Previous studies involving nasal mucosal imprints on plastic strips and nasal biopsy specimens obtained before and during the birch-pollen season

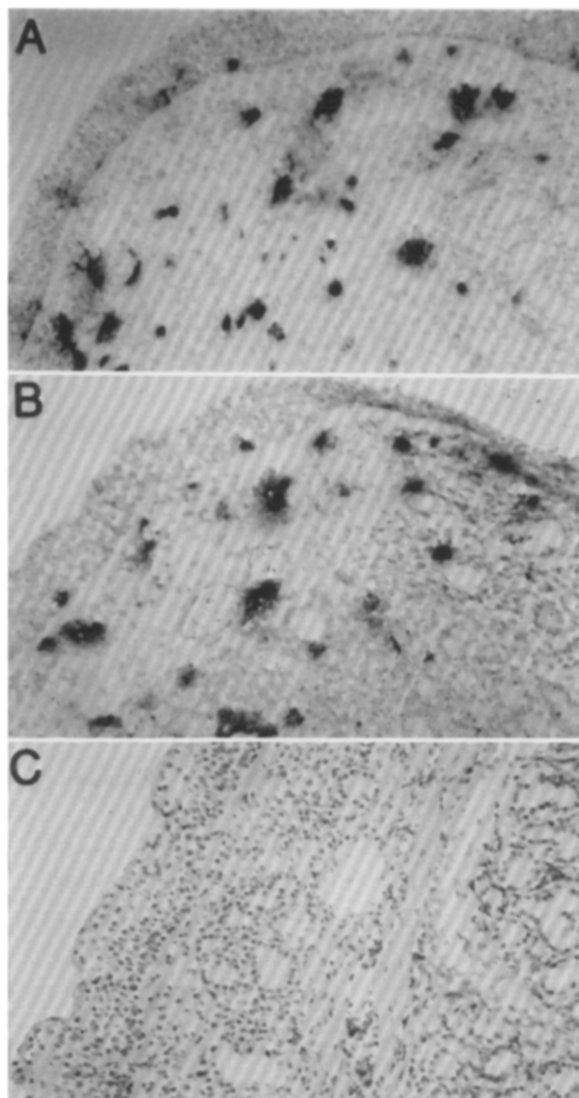


FIG. 3. Immunostaining patterns in the nasal mucosa. **A**, Activated eosinophils in nasal submucosa and within epithelium (alkaline-phosphatase anti-alkaline-phosphatase method). **B**, Mast cells of both subtypes (MC_T s and MC_{TC} s) in submucosa and MC_T s within the epithelium. **C**, Negative control section with primary antibody layer omitted.

support this view.^{26, 27} Alternatively, mast cell precursors may home in on the nasal mucosa and proliferate in the epithelium during the season.⁴

Our findings of local tissue eosinophilia and an increase in the number of ECP-secreting eosinophils are consistent with previous studies of nasal washings that have demonstrated increases in eosinophils. This was also true for ECP concentrations after both nasal allergen provocation^{3, 28} and during the pollen season.^{29, 30} Taken together, these observations provide clear evidence that nasal-allergen exposure results in

both the local accumulation of eosinophils and, more importantly, activation of eosinophils for release of their granule products.

In summary, we have provided direct evidence from nasal biopsy specimens for local tissue eosinophilia and epithelial migration of MC_T s in grass pollen-sensitive subjects during seasonal allergen exposure. Our findings support a role for migration of MC_T and eosinophil activation in the pathogenesis of seasonal AR.

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