

Selective recruitment of eosinophils by substance P after repeated allergen exposure in allergic rhinitis

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We have investigated the nasal response to substance P after pollen exposure in seasonal allergic rhinitic patients. Seven patients with strictly seasonal allergic rhinitis were studied during the pollen season, 24 h after nasal challenge with pollen. They received increasing doses of nebulized substance P (0 to 80 nmol) in each nostril. Responses were assessed by measurement of nasal airway resistance by posterior rhinomanometry and quantification of albumin, histamine, and inflammatory cells in the nasal lavage fluid. Nasal airway resistance increased in a dose-dependent manner after substance P challenge. Protein and albumin in nasal lavage fluids increased after administration of substance P: from 2.6 ± 0.3 to 6.8 ± 1.1 mg for protein ($P < 0.01$) and from 0.2 ± 0.1 to 3.1 ± 0.6 mg for albumin ($P < 0.02$). Expressed as a percentage of total protein, albumin increased from $10.5 \pm 3.6\%$ to $39.9 \pm 3.5\%$ ($P < 0.02$), suggesting occurrence of plasma leakage. No histamine release was observed after challenge with substance P. Total cell counts significantly increased from 11.4 ± 2.4 to $41.8 \pm 17.3 \times 10^3$ cells/ml after substance P ($P < 0.05$). Eosinophils were already numerous before substance P challenge ($2.1 \pm 0.7 \times 10^3$ cells/ml), and the number of eosinophils markedly increased in all patients after substance P (for the whole group, 25.8 ± 13.3 cells/ml, $P < 0.05$). In contrast, the number of neutrophils only slightly increased in five patients, and changes did not reach significance for the group as a whole. Our results show that substance P induces nasal obstruction and albumin extrusion in allergic rhinitic patients after repeated pollen exposure. These vascular phenomena are associated with recruitment of eosinophils. Since substance P is known to be released after nasal allergen challenge, our data suggest a role for substance P in the chronic eosinophilic inflammation of the nasal mucosa observed in symptomatic allergic rhinitis.

In seasonal allergic rhinitis, repeated natural or experimental exposure to allergen induces eosinophilic infiltration of the nasal mucosa, which is greater than that observed after a single exposure to allergen (2, 11, 17). The occurrence of an increase in nonspecific nasal responsiveness to histamine has been described after experimental allergen challenge (21). This includes an increase in the clinical response to histamine, which is correlated with the presence of allergen-induced eosinophilic inflammation of the nasal mucosa. These data suggest that nasal inflammation, consisting mainly of an infiltration of eosinophils, plays a critical role in allergen-induced increase in specific and nonspecific nasal responsiveness.

Substance P is an undecapeptide localized in sensory C-fibers in the upper airways of animals and man (1, 20). In patients with allergic rhinitis, substance P is released in nasal lavage fluid after experimental allergen challenge (14, 15). In asymptomatic allergic rhinitic patients, substance P administered by intranasal nebulization induces nasal obstruction, whereas no such effect is observed in control subjects (6). Substance P-induced nasal obstruction is associated with a proinflammatory effect involving microvascular leakage and recruitment of inflammatory cells, mainly neutrophils and occasionally eosinophils, in the nasal lumen (4).

Since specific and nonspecific nasal responsiveness is modified after nasal allergen exposure, we

investigated the nasal response to substance P after pollen exposure in strictly seasonal allergic rhinitic patients. Nasal obstruction and inflammatory changes, such as plasma leakage and inflammatory cell recruitment, were studied.

Material and methods

Subjects

Seven patients (mean $\pm$ SD: 26 ± 2 years; five men and two women) with strictly seasonal allergic rhinitis were included in the study. All had a clinical history of sneezing, secretions, and nasal obstruction after natural exposure to pollen. Skin prick tests were positive for mixed grass pollen (Stallergènes, Paris, France) and negative for all other common allergens (house-dust mite, cat and dog dander, and molds). Nasal allergy to grass pollen was confirmed by an early response to nasal pollen challenge. Criteria for a positive challenge were occurrence of nasal symptoms and at least a 100% increase in nasal airway resistance (NAR) within 15 min after intranasal nebulization of mixed grass pollen (5 μ g diluted in normal saline per nostril) (Stallergènes, Paris, France). Patients had been asked to stop antihistamine medication 1 week before the experiment and to abstain from taking any other medication during the 2 months prior to the study. They were informed of the risks and purpose of the investigation and gave their written consent to participate. The study was approved by the ethics committee of our hospital.

Study design

The study was conducted during the pollen season (May–July). For each patient, grass pollen counts were monitored during the 3 weeks prior to the study day with a volumetric Hirst type trap (Lanzoni VPPS 2000) (10). Patients were included in the experiment at least 7 days after the beginning of their seasonal allergic nasal symptoms. A nasal challenge with mixed grass pollen was performed on the day of inclusion. Twenty-four hours later, the substance P challenge was performed as previously described (4). Briefly, initial NAR was measured. Four prechallenge nasal lavages were performed with saline (NaCl 0.9%; 37°C) and NAR was again measured. The vehicle (NaCl 0.9%) was first nebulized, and NAR was measured, followed by a fifth nasal lavage. Increasing doses of substance P (2.5, 5, 10, 20, 40, and 80 nmol) were nebulized every 8 min into each nostril from freshly prepared solutions of substance P. Each nebulization was given with a plastic hand-held nebulizer (100 ± 3 μ l per puff; Stallergènes, Paris, France) while subjects held their breath. Clinical responses (sneezing, secretions, etc.) were as-

sessed after each dose of substance P. NAR was measured 1.5, 3, 5, and 7.5 min after each dose of substance P. The nose was lavaged again 10 min after the last dose.

Assessment of the response

NAR was measured by posterior rhinomanometry (4, 8). Briefly, the subjects breathed through the nose into a mask covering the nose. Nasal airflow was measured with a pneumotachograph (no. 1, Fleisch, Lausanne, Switzerland) attached to the mask and connected to a differential pressure transducer (Validyne Model MP 45-1-1871; Validyne Corp., Northridge, CA, USA). Transnasal pressure was the difference between pressure in the mask and at the top of a plastic catheter placed over the tongue into the oropharynx, measured with an identical differential pressure transducer. Airflow and transnasal pressure measured at 10-ms intervals between the onset of inspiration and maximal inspiratory airflow were monitored and fed in parallel into a microcomputer. NAR was calculated at each airflow and the linear regression of NAR as a function of airflow was determined (Mediprom rhinomanometer, Paris, France). NAR was calculated by interpolation at an airflow of 150 ml/s.

Nasal lavages were performed with saline (NaCl 0.9%; 37°C). Five 1-ml aliquots were injected into each nostril past the vestibule, with a soft-tipped catheter. After each injection, subjects expelled the fluid by gently blowing their noses into a plastic tube. The volume of the recovered fluid was measured.

The vortexed fluid (2 ml) was centrifuged at 1300 g for 10 min (Beckman TJ-6 centrifuge, Beckman Instruments, Fullerton, CA, USA). The supernatant (1 ml) was sonicated and used for protein analysis by the Coomassie brilliant blue technique (12) and for measurement of albumin by nephelometry (Behring, Rueil-Malmaison, France). Perchloric acid (1 ml, 0.8 N) was added to the remaining supernatant. The tubes were stored at -20°C until histamine was measured by an automated fluorometric technique with a detection limit of 0.5 ng/ml (18).

The lavage fluid (2 ml) was aliquoted for cytologic analysis. Total cell count was performed with a Malassez cell. After centrifugation at 850 g for 5 min, cells were resuspended in Hanks' solution and cyto-centrifuged for 5 min at 550 rpm (Shandon cytopspin, Shandon, Pittsburgh, PA, USA), processing 30 000 cells per slide. The cells were air-fixed before staining with Diff-Quick® (Merz + Dade AG, Düringen, Switzerland). Cells were identified according to standard cytologic criteria, and differential cell counts were based upon examination of 400 cells.

Expression of results and statistical analyses

The highest NAR value ($\text{cmH}_2\text{O.s.l}^{-1}$) measured after each dose of substance P was used to construct the dose-response curves. Biochemical and cytologic analyses were performed on the nasal lavages performed before and after saline and after substance P challenge. Results are expressed as group average $\pm$ SEM. Comparisons between pre- and post-substance P data were made by nonparametric analyses of variance for nasal airway resistance and by the Wilcoxon rank test for the other parameters. A *P* value of less than 5% was considered significant.

Results*Pollen exposure and nasal inflammation before substance P challenge*

Grass pollen counts were at least 44 grains/ m^3 per week during the preceding 3 weeks for each patient studied. Most of the counts were greater than 100 grains/ m^3 per week; for two patients, more than 600 grains/ m^3 per week were detected. This indicates a high level of grass pollen grains in the atmosphere during the 3 weeks preceding the substance P challenge, since 30–50 grains/ m^3 per week is usually associated with nasal symptoms of allergic rhinitis (5).

On the substance P day, before saline and substance P challenge, the eosinophil count was $3.9 \pm 1.5 \times 10^3$ cells/ml of nasal lavage fluid and accounted for $13.2 \pm 4.2\%$ of total cells. This result indicates the presence of an inflammatory state of the nasal mucosa, since out of pollen exposure, very few eosinophils are commonly observed (2, 4, 11).

Responses to substance P

No parameter was modified after saline challenge. Therefore, baseline levels used for evaluation of the effect of substance P were values obtained after saline challenge.

Nasal airway resistance. Substance P induced a significant and dose-dependent increase in NAR (Fig. 1). The increase in NAR was already significant at the first dose used (2.5 nmol) compared with baseline ($P < 0.05$). Mean maximal increase was 4.9-fold basal NAR (range: 3.5- to 13.8-fold).

Clinical responses. Substance P induced sneezing in three subjects after the first dose of substance P. Small amounts of spontaneously dripping secretions occurred in two patients. Substance P induced a dose-dependent flush starting within 30–45 s and disappearing within 3 min. The sensation of warmth

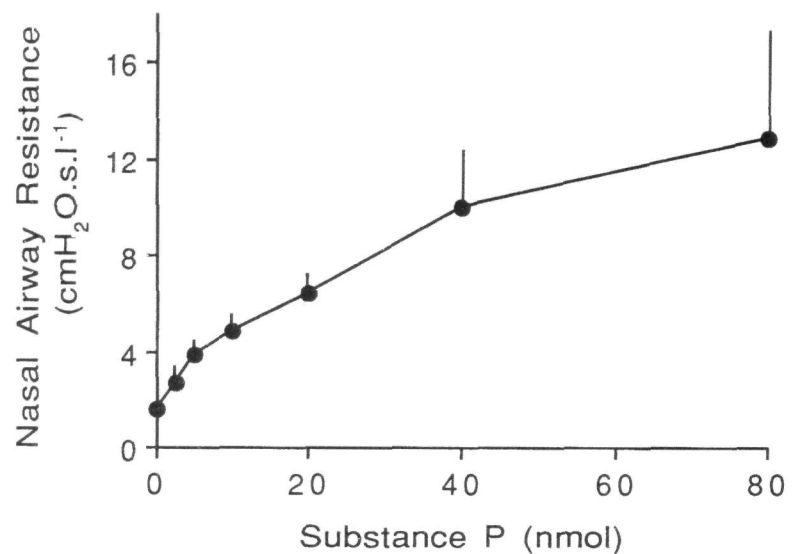


Fig. 1. Dose-response curve of effect of substance P nasal airway resistance on patients with allergic rhinitis during pollen exposure. Points are means of seven experiments. Bars represent SEM.

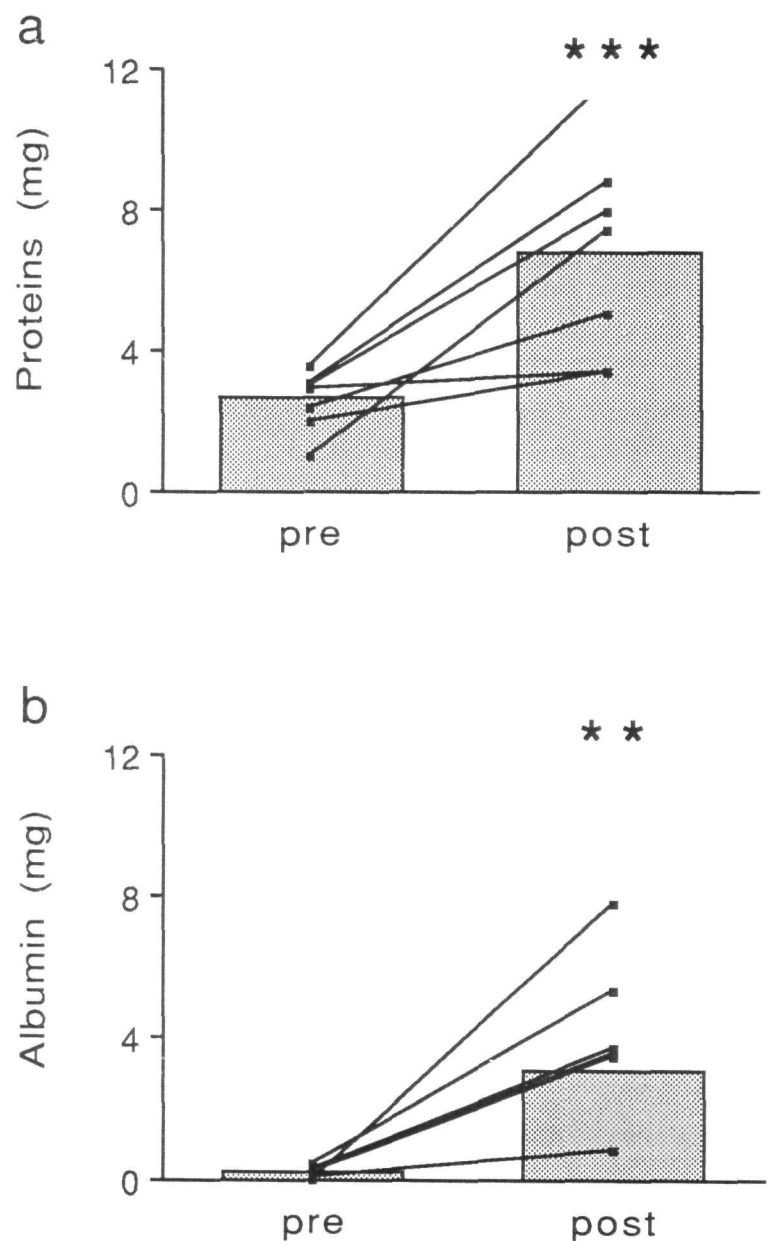


Fig. 2. Effect of substance P on release of proteins (a) and albumin (b) in nasal lavage fluid during pollen exposure. Individual values before (pre) and after (post) substance P challenge in seven patients with allergic rhinitis to pollen are indicated by individual points. Means are represented by gray blocks. ** $P < 0.02$; *** $P < 0.01$.

began in the cheeks and ears and, as the dose increased, reached the neck, arms, chest, and legs. The intensity of the reaction was so pronounced in two patients that the challenge had to be stopped at 40 nmol substance P.

Biochemical responses. Substance P induced a significant increase in protein extrusion in nasal lavage fluid, from 2.6 ± 0.3 to 6.8 ± 1.1 mg ($P < 0.01$)

(Fig. 2a). Albumin also increased from 0.2 ± 0.1 mg to 3.1 ± 0.6 mg ($P < 0.02$) (Fig. 2b). Expressed as a percentage of total protein, albumin increased from $10.5 \pm 3.6\%$ to $39.9 \pm 3.5\%$ ($P < 0.02$). No significant increase in histamine recovery was observed: 60.0 ± 19.3 ng before and 69.0 ± 26.2 ng after substance P challenge.

Cellular responses. Substance P induced a significant increase in total cells in nasal lavage fluid: 11.4 ± 2.4 before and $41.8 \pm 17.3 \times 10^3$ cells/ml after substance P administration ($P < 0.05$) (Fig. 3a).

The neutrophil count increased in five patients and decreased in two. Changes did not reach significance for the group as a whole: 1.5 ± 0.4 before and $6.5 \pm 3.6 \times 10^3$ cells/ml after substance P. Expressed as a percentage of total cell count, neutrophils accounted for $11.9 \pm 2.5\%$ before and $19.0 \pm 6.7\%$ after substance P (Fig. 3b).

After substance P challenge, the eosinophil count increased in all subjects, and for the group, the increase was statistically significant: from 2.1 ± 0.7 before to $25.8 \pm 13.3 \times 10^3$ cells/ml after substance P challenge ($P < 0.05$). Expressed as a percentage of total cells, the number of eosinophils significantly increased from $17.4 \pm 16.6\%$ to $42.7 \pm 6.4\%$ ($P < 0.02$) (Fig. 3c).

Lymphocytes were rare and accounted for less than 0.5% of total cell count in all samples.

Epithelial cell counts were not significantly modified by substance P: 7.7 ± 2.0 before and $12.6 \pm 4.2 \times 10^3$ cells/ml after substance P.

Discussion

Our results show that exogenous substance P challenge after repeated allergen exposure during seasonal allergic rhinitis induces recruitment of a large number of eosinophils in the nasal lavage fluid. This is associated with nasal obstruction and extrusion of albumin, due to vascular events. No histamine release was observed after substance P challenge, indicating an absence of mast-cell activation.

Nasal obstruction and extrusion of albumin were observed on challenge with substance P during pollen exposure. These vascular events had been described previously, when substance P challenge was administered out of pollen exposure in asymptomatic allergic rhinitic patients (4). They were not associated with any release of histamine in nasal lavage fluid either during (this study) or out of pollen exposure (3, 4). Hence, the lack of activation of human nasal mast cells by substance P that was previously suggested was also observed during nasal inflammation induced by repeated pollen exposure. The clinical (vasodilation) and biochemical (albumin extrusion) responses to substance P after repeated

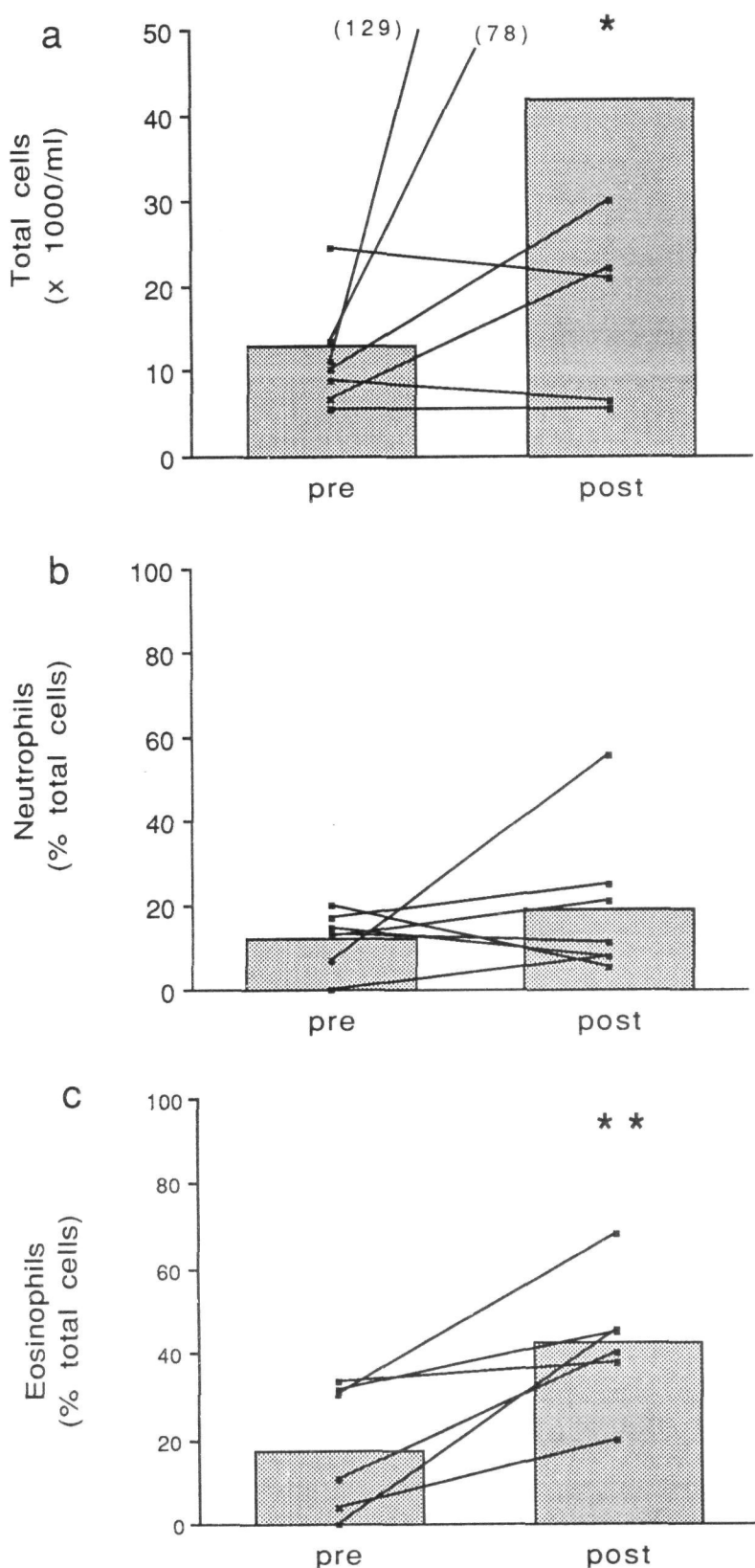


Fig. 3. Effect of substance P on cell output in nasal lavage fluid during pollen exposure. Individual values of total cells (a) and percentage of neutrophils (b) and eosinophils (c) before (pre) and after (post) substance P challenge in seven patients with allergic rhinitis to pollen are indicated by individual points. Means are represented by gray blocks. * $P < 0.05$; ** $P < 0.02$.

pollen exposure were identical to those found out of the pollen season, although of greater intensity during pollen exposure (4). This suggests that similar vascular events are induced by substance P in the nose, whether or not the nasal mucosa is inflamed.

During pollen exposure, after substance P nebulization, a large number of eosinophils were recruited in the nasal lavage fluid. This massive recruitment of eosinophils is in contrast with our previous finding that substance P was administered outside pollen exposure in patients free of symptoms (4): under similar methods, eosinophil recruitment was not consistent in all subjects and was always of low magnitude. Our present results suggest that, in the presence of nasal eosinophilic inflammation, substance P has an enhanced chemokinetic effect for eosinophils. However, it is likely that a large number of eosinophils had already been infiltrating the nasal mucosa and/or were marginated on the vascular walls. This is suggested by the presence of eosinophils in nasal lavages performed before any challenge during pollen exposure. It is further supported in the literature by the fact that nasal biopsies taken during and out of the pollen season showed a seasonal increase in eosinophils in the submucosa and the nasal epithelium (2, 11). Therefore, our study suggests that substance P induces migration of eosinophils from the submucosa across the epithelial barrier into the nasal lumen. This is supported by the demonstration that, in allergic subjects, substance P potentiates chemotaxis of blood eosinophils induced by platelet-activating factor *in vitro* (16), whose metabolism has been described after nasal allergen challenge (13). Other mechanisms could account for eosinophil recruitment by substance P during inflammation of the nasal mucosa, such as increased expression of substance P binding sites on infiltrated eosinophils, release of secondary mediators with chemotactic activity for eosinophils, or an effect of substance P on the expression of adhesion molecules on eosinophils and/or endothelial cells (19).

For neutrophils, no significant increase in number in nasal lavage fluid was induced by substance P during pollen exposure. This is in contrast with our previous report performed outside antigen exposure, where a marked recruitment of neutrophils occurred after challenge with substance P (4). Controversy exists regarding the importance of the neutrophil as an effector cell in chronic allergic inflammation (7, 9). Results in symptomatic allergic rhinitis (this report and 2, 11) agree with findings in similar diseases such as chronic sinusitis and nasal polyposis where sparse neutrophils and marked eosinophilic infiltration are observed (7). This supports a major role for eosinophils in the pathogenesis of continuous allergic mucosal disease and a lesser role for neutrophils.

In conclusion, we have found that substance P

induces a massive eosinophil recruitment when administered in the nose in the presence of nasal inflammation induced by repeated allergen exposure. This, together with the recent report of release of substance P in nasal lavage fluid after allergen challenge (14, 15), suggests that substance P might contribute to the eosinophilic inflammation of the nasal mucosa observed in allergic rhinitis and might play a role in continuous nasal allergic disease.

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