

Original article

Changes in airway inflammation following nasal allergic challenge in patients with seasonal rhinitis

Background: Seasonal allergic rhinitis could predispose to the development of chronic bronchial inflammation as observed in asthma. However, direct links between nasal inflammation, bronchial inflammation and airway responsiveness in patients with seasonal allergic rhinitis and without asthma are not fully understood. The aim of this study was to analyse the changes induced by allergic nasal challenge outside the pollen season in airway responsiveness and bronchial inflammation of patients with seasonal allergic rhinitis.

Methods: Nine patients were evaluated after either grass pollens or placebo nasal challenge in a randomized cross-over double-blinded trial. Nasal parameters were recorded hourly and airway responsiveness was assessed by methacholine challenge. Cytological examinations and cytokine measurements were performed in nasal lavage and induced sputum. Eosinophil activation was investigated by eosinophil-cationic protein expression and secretion.

Results: Airway responsiveness was increased after allergic nasal challenge. Total eosinophils and eosinophils expressing eosinophil-cationic protein were increased in induced sputum after allergic nasal challenge. Both eosinophil number and eosinophil-cationic protein concentration in induced sputum were correlated to methacholine responsiveness.

Conclusions: These results suggest that eosinophils participate to the bronchial inflammation in patients with seasonal allergic rhinitis following allergic nasal challenge outside the pollen season and might explain changes in airway responsiveness.

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Background

Epidemiological and pathophysiological studies suggest that asthma and allergic rhinitis are closely related diseases (1–3). They often occur together and allergic rhinitis might increase the risk of asthma development (1, 4–6). Asthma and allergic rhinitis might be the two manifestations of one common allergic respiratory syndrome (2–7). However, the relationship between asthma and allergic rhinitis is complex and upper and lower airways might interact with each other.

Chronic bronchial inflammation and hyperresponsiveness are major determinants in the pathogenesis of asthma. The presence of bronchial inflammation in nonasthmatic patients with seasonal allergic rhinitis has been already reported (8–10). Natural exposure to pollen and nasal or endobronchial allergen challenge induce

inflammatory cell recruitment and interleukin (IL)-5 expression leading to bronchial eosinophilic inflammation in these nonasthmatic patients with allergic rhinitis (11–13). Braunstahl et al. (14, 15) also found increased inflammatory markers in nasal mucosa from nonasthmatic patients with seasonal allergic rhinitis after segmental bronchial allergen provocation, suggesting a systemic cross talk between nasal and lower airways.

High incidence of airway hyperresponsiveness in nonasthmatic patients with seasonal allergic rhinitis is also well known (16–19). Natural exposure to pollen and nasal allergen challenge induce an increase in airway responsiveness to methacholine in nonasthmatic patients with seasonal allergic rhinitis (2, 8, 12).

Although the relationship between seasonal allergic rhinitis and asthma has been well established in epidemiologic and clinical studies, direct links between nasal, bronchial inflammation and airway hyperresponsiveness have not been evidenced in seasonal allergic rhinitis without asthma.

Indeed, although several authors have investigated the effect of natural exposure or nasal challenge to grass pollens on lower airways (9–13, 20–22), the relationship

Abbreviations: PD20 methacholine, provocative dose in mg causing a 20% decrease in forced expiratory volume in 1 s; IR, index of reactivity (concentration of allergen that induces a 7 mm wheal in a group of sensitized individuals); EG2+ eosinophils, eosinophils expressing eosinophil-cationic protein; NBI, nasal blockage index.

between bronchial inflammation and airway hyperresponsiveness induced by controlled nasal provocations in nonasthmatic patients with allergic rhinitis outside the pollen season has not been evaluated in a cross-over design.

The aim of this study was to analyse the changes induced by allergic nasal challenge outside the pollen season in bronchial inflammation and airway hyperresponsiveness in patients with seasonal allergic rhinitis and without asthma.

Material and methods

Patients

Nine caucasian volunteers (Table 1) with history of recurrent seasonal pollen-induced rhinitis and bronchial hyperresponsiveness to inhaled methacholine (methacholine PD20: median 0.44 mg; range 0.05–4 mg) outside the pollen season participated in the study. None of the patients had asthma-related respiratory symptoms according to international asthma guidelines (23). All had positive allergic skin prick test responses to grass pollens, six patients were also positive to house dust mite and/or cat (Table 1). All volunteers were nonsmokers who had no other respiratory conditions apart from their allergic rhinitis.

All patients had normal pulmonary function test and no bronchial reactivity to 400 µg salbutamol (Table 1).

Study design

This was a randomized, cross-over, double-blind study with two interventions and two periods outside the pollen season (Fig. 1). The study was performed between October 2002 and February 2003. We obtained approval from local ethical committee, and written informed consent.

Each patient was studied on 2 days, each separated by 2 weeks. The tests were always conducted at the same times of the day (Fig. 1).

The design of the study was done by assuming that allergenic challenge increases bronchial responsiveness to methacholine and eosinophil number in induced sputum compared with placebo from previously published data (10, 24–26). The criterion for significance α was set at 0.05 and the power at 0.80, giving an

Placebo or Grass pollens

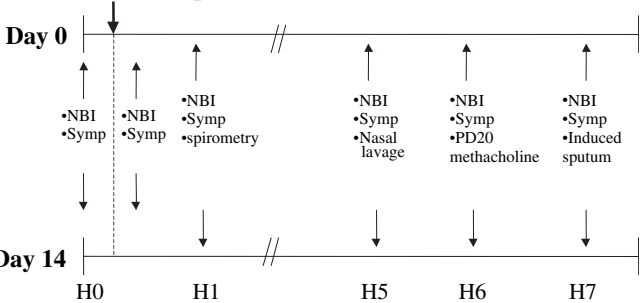


Figure 1. Study protocol. NBI: nasal blockage index; symp: nasal symptom score; PD20 methacholine: provocative dose in mg causing a 20% decrease in forced expiratory volume in 1 s. H0 = 8 AM; H1 = 9 AM; H5 = 1 PM; H6 = 2 PM; H7 = 3 PM.

estimated sample size of six subjects to which 50% was added (three subjects) for expected attrition owing to unsuccessful sputum induction.

Nasal challenge

Hundred microlitres of allergens or placebo (five grass pollens or diluent; Stallergenes®, Stallergène SA, Antony, France) were delivered as previously described (27) into both nostrils at total lung capacity during breath holding and the nose was blocked with a nose-clip for 10 min afterwards to avoid bronchial penetration. Patients were advised to keep their head in a forward prone position to avoid swallowing or aspiration of allergens or nasal fluids to the lower respiratory tract. Nasal blockage index [NBI = (oral peak flow rate – nasal peak flow rate)/oral peak flow rate] (28) and nasal symptom score [sneezing 0, 0 point; 1–3, 1; > 3, 2; rhinorrhea: none, 0; mild, 1; abundant, 2; congestion: none, 0; mild, 1; complete (bilateral), 2; pruritus: none, 0; mild (eyes or throat), 1; severe (eyes or throat), 2] were determined at baseline, 10 min after the diluent, 10 min after each allergen increasing dose [0.2, 0.5, 0.8, 1.8, 2.8 index of reactivity (IR)] and 1, 5, 6 and 7 h after nasal challenge. The challenge solution was administered every 10 min until the NBI had increased by at least 60% compared to the diluent or the fifth dose of solution was administered. Nasal symptom score ≥ 4 was considered as a positive response.

Table 1. Characteristics of the patients

Patients	Age (years)	Sex	Skin prick test to grass pollen (mm ²)	FEV ₁ prebronchodilator (% predicted)	FEV ₁ postbronchodilator (% predicted)	FVC (% predicted)
1	24	M	45*	98	98	113
2	21	M	36	97	99	112
3	34	F	105*	94	97	95
4	26	F	63*	103	102	100
5	24	M	30*	99	102	104
6	28	F	25*	107	98	120
7	43	F	64*	98	102	104
8	32	F	49	103	98	114
9	33	M	50	108	101	108
Median	28	4M/5F	49	99	99	108

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

*Patient also positive to house dust mite and/or cat.

Pulmonary function and methacholine-challenge testing

Flow-volume curves were obtained with a Biomedin spirometer (Biomedin srl, Padova, Italy). The spirometry technique met international standards and reference values were those of the European Respiratory Society (29). Airway responsiveness was assessed by methacholine challenge testing with an automatic inhalation-synchronized Mefar MB3 dosimeter jet nebulizer (Mefar srl, Bovezzi, Italy) as previously described (30). The methacholine provocative dose causing a 20% decrease (PD₂₀ methacholine) in forced expiratory volume in 1 s (FEV₁) from control FEV₁ was determined by interpolation from the dose-response curve (30).

Nasal lavage and sputum induction

Nasal lavage was performed with the patients keeping their head in a forward prone position and soft palate closed as previously described (27). The nasal fluids were immediately centrifuged and the supernatant was aspirated and stored at -70°C in the presence of aprotinin. Cells were resuspended and a total cell count was performed. Differential cell counts were performed on May-Grünwald-Giemsa stained slide if possible.

Sputum induction was performed with an aerosol of hypertonic saline using the method of Pin et al. (31). The sputum was examined within 1 h with a modified method described by Pizzichini et al. (32). The final suspension was centrifuged and the supernatant was aspirated and stored at -70°C in the presence of aprotinin. Total nonsquamous cell count was performed in a hemocytometer and expressed as millions per milligram of selected induced sputum. The proportion of salivary squamous cells was noted and cell viability was determined by the trypan blue exclusion method. From the remainder of the selected induced sputum, 10 cytopins were prepared, air-dried, fixed and stained as required. Differential cell counts were performed by counting 400 cells on May-Grünwald-Giemsa stained slides. Another two slides were stained with toluidine blue and 1000 cells were counted for metachromatic cells. Results were expressed as a percentage of the total nonsquamous count. For immunocytochemical staining, the remaining slides were fixed in acetone and stored at -20°C until use. Slides were coded and cell count was performed by an expert observer who did not know the clinical characteristics of the patients. Only samples with cell viability > 70% and squamous cell contamination < 20% were considered adequate.

Measurement of inflammatory markers in nasal lavage and sputum supernatant

Nasal fluid and sputum supernatant concentrations of IL-4, IL-5, IL-10, GM-CSF, MCP-1, TNF- α , IFN- γ and eotaxin-1 were measured by commercially available ELISAs (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The lower detection limits of the assays were: 0.13 pg/ml (IL-4), 3 pg/ml (IL-5), 0.5 pg/ml (IL-10), 0.25 pg/ml (GM-CSF), 5 pg/ml (MCP-1), 0.12 pg/ml (TNF- α), 8 pg/ml (IFN- γ), and 5 pg/ml (eotaxin-1).

Nasal and sputum eosinophil-cationic protein were measured by a commercially available enzyme assay (CAP-FEIA; Pharmacia, St Quentin-enYvelines, France), with a lower detection limit of 2 ng/ml.

Immunocytochemistry

Eosinophil activation was investigated by immunocyto-chemistry by using a monoclonal antibody (EG2 clone; Pharmacia) specific

for human eosinophil-cationic protein. An isotype-matched control antibody (MPOC-21; Sigma Chemical Co., St Louis, MO, USA) was used as control.

Statistical analysis

Results are presented as median and range. The Wilcoxon's paired test was used for comparisons. Correlations were assessed by the Spearman's rank order test. A *P* value < 0.05 was regarded as significant.

Results

All nine subjects who entered the protocol completed the study. Nasal blockage index was significantly increased at the end of nasal challenge with grass pollens compared to either baseline (H0) and placebo in the group of nine patients (Fig. 2). Nasal symptom score was also significantly increased at the end of allergen nasal challenge and at 9 AM (H1) compared to either baseline (H0) or placebo (Fig. 2). None of the patients had nasal symptom score ≥ 4 or $\geq 60\%$ increase in nasal blockage index compared to diluent after placebo nasal challenge.

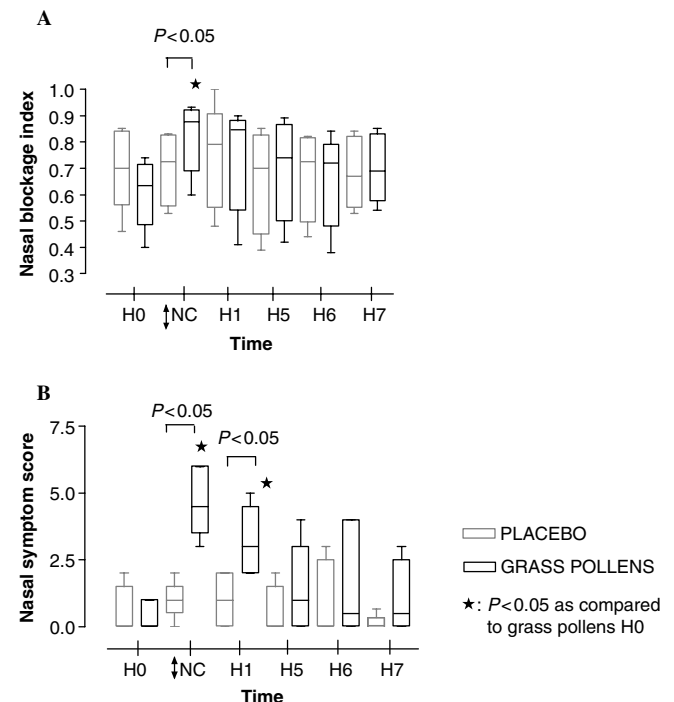


Figure 2. Effect of nasal challenge on nasal parameters. (A) Nasal blockage index; (B) Nasal symptom score. Box and whiskers plot with median, interquartile range and minimum and maximum values; grey box: placebo; black box: grass pollens; H0 = 8 AM; $\downarrow$ NC: end of nasal challenge; H1 = 9 AM; H5 = 1 PM; H6 = 2 PM; H7 = 3 PM; $\star$: *P* < 0.05 as compared to grass pollens.

Less than 20 000 cells were obtained in nasal lavage performed at 1 PM (H5) in agreement with the lack of modification in nasal blockage index and symptom score compared to baseline. No difference in total cell counts was observed between allergen and placebo-challenge day.

Lower airway responsiveness to methacholine was significantly increased after nasal challenge with grass pollens compared to placebo ($P = 0.03$, Fig. 3). The increase in bronchial airway responsiveness after allergic nasal challenge was not associated with change in nasal parameters.

Squamous cell contamination >20% was found in sputum from three patients at day 0 and/or 14 (the corresponding samples were excluded). Total nonsquamous cell count/mg sputum was not different between allergen and placebo nasal challenge-day. Neutrophils, lymphocytes, metachromatic and epithelial cells, either expressed as total cell number or relative proportion, were not modified in induced sputum after allergic nasal challenge as compared with placebo. In contrast, eosinophil number was increased [2.9 (0–30.8)% to 32.3 (1.2–57.3)%, $P = 0.02$] and macrophage number was decreased [31.1 (15.2–57.3)% to 19.2 (1.6–51.4)%, $P = 0.03$] in induced sputum after allergic nasal challenge as compared with placebo (Figs 4 and 5). Eosinophils expressing eosinophil-cationic protein (EG2+ eosinophils) were increased [5.9 (1.1–13.8)% to 21.4 (2.4–53.4)%, $P = 0.03$] in induced sputum after allergic nasal challenge as compared with placebo (Fig. 5C,D).

IL-4, GM-CSF and eotaxin-1 were under the detection limit of the assay in nasal fluids and sputum supernatants. There was an increase in eosinophil-cationic protein concentration in sputum supernatant but not in nasal

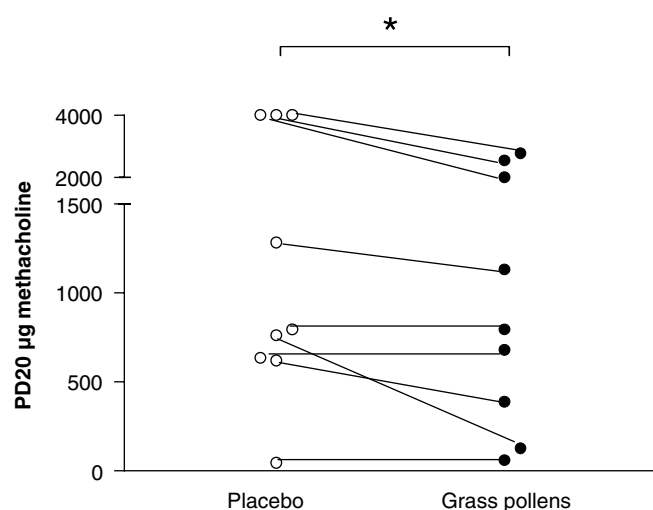


Figure 3. Increase in airway responsiveness to methacholine after nasal allergic challenge. Individual values are presented. PD20 methacholine: provocative dose in mg causing a 20% decrease in FEV₁. * $P < 0.05$.

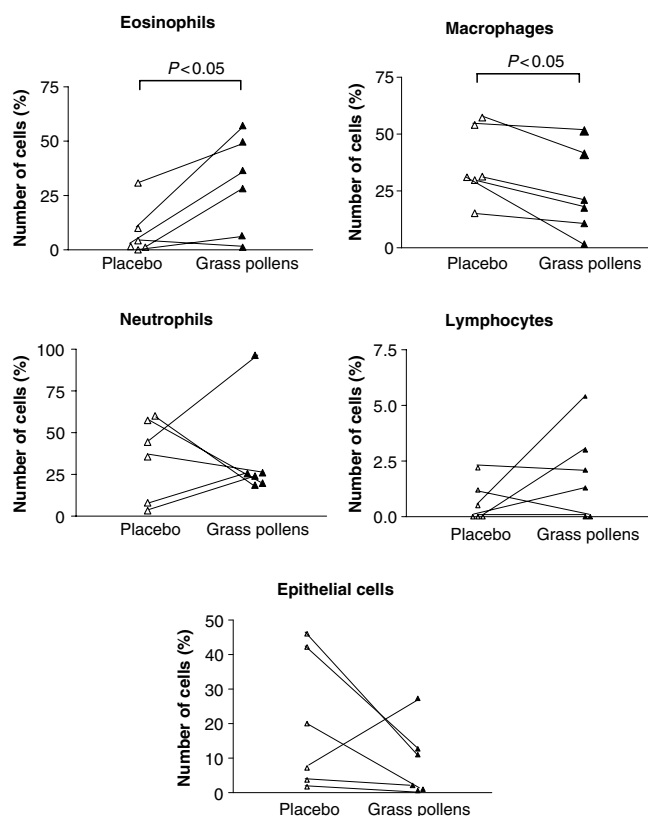


Figure 4. Differential cell counts in induced sputum. Number of cells was expressed as a percentage of the total nonsquamous cell count. Individual values are presented.

fluids after allergic nasal challenge compared to placebo, although the changes did not reach statistical significance [105 (12–130) $\mu\text{g/l}$ to 135 (23–200) $\mu\text{g/l}$, $P = 0.06$]. A decrease in IL-10 concentration was observed in the same conditions in sputum supernatant [2.6 (0–5.5) pg/ml to 0.25 (0–3.2) pg/ml , $P = 0.06$]. No other difference in cytokine concentrations was observed between allergenic challenge and placebo in nasal fluid or sputum supernatant (data not shown). IL-5 in induced sputum increased in three of six patients after nasal allergic challenge (0–11, 6–10, 0–6 pg/ml), whereas the others remained undetectable.

An inverse correlation between the cumulative dose of allergen given with nasal challenge [1.6 (0.4–5.6) IR] and the number of eosinophils in induced sputum was observed but did not reach statistical significance ($r = -0.8$, $P = 0.06$, $n = 6$). However, no correlation was found between the cumulative dose of allergen given with nasal challenge and EG2+ eosinophils number or eosinophil-cationic protein concentration in sputum supernatant.

The increase in airway responsiveness was not associated with changes in nasal blockage index, symptom score or cellularity. However, a correlation between methacholine PD20 after nasal allergic challenge and the cumulative dose of allergen [1.6 (0.4–5.6) IR] was found ($r = 0.70$, $P = 0.04$, $n = 9$). Inverse correlations between methachol-

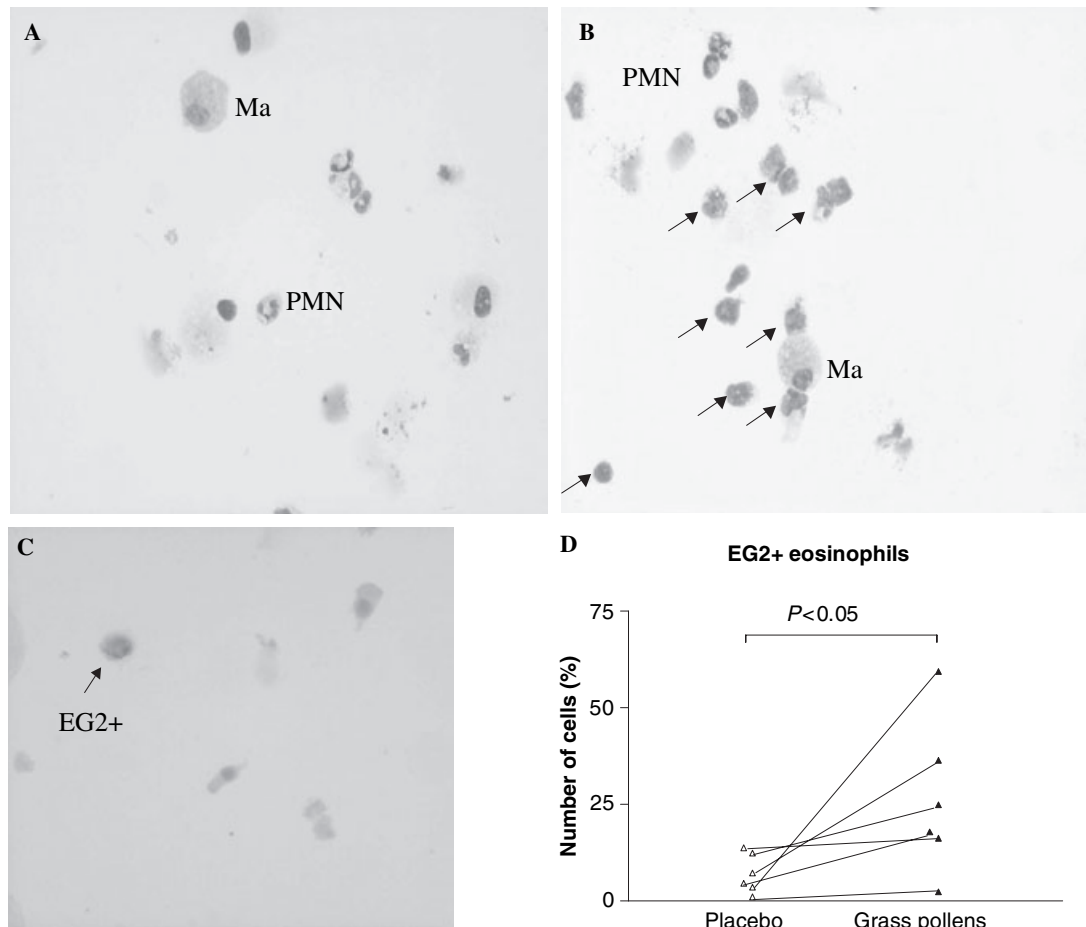


Figure 5. Increase in eosinophils after allergic nasal challenge in induced sputum. May–Grünwald–Giemsa stained cytocentrifuged sputum cells (patient no. 4): (A) placebo; (B) grass pollens. $\rightarrow$: eosinophils, Ma: macrophages, PMN: neutrophils. Activation of eosinophils after allergic nasal challenge in induced sputum: (C) EG2-immunostained eosinophils are increased after allergic nasal challenge; (D) eosinophils expressing eosinophil-cationic protein (EG2+ eosinophils) are increased in induced sputum after nasal challenge with grass pollens compared to placebo.

line PD20 after nasal challenge (placebo $n = 6$ and grass pollens $n = 6$) and number of eosinophils in induced sputum or eosinophil-cationic protein concentration in sputum supernatant were observed ($r = -0.59$, $P = 0.04$, $n = 12$ and $r = -0.81$, $P = 0.001$, $n = 12$ respectively). No correlation between EG2+ eosinophils number and methacholine PD20 was found.

Discussion

To our knowledge, this is the first diluent controlled cross-over study demonstrating that a nasal allergic challenge induces an increase in bronchial airway responsiveness and eosinophilic inflammation in nonasthmatic patients with seasonal allergic rhinitis outside the pollen season. Chakir et al. have compared bronchial inflammation in biopsies from eight nonasthmatic patients with seasonal rhinitis both in and out the pollen season (12).

They found that natural pollen exposure was associated with an increase in the number of bronchial eosinophils and airway responsiveness to methacholine during the pollen season. However, they did not find any correlation between changes in inflammatory cell counts and changes in methacholine PC20 values. This could be explained by the fact that during natural allergen exposure, as in the population studied by Chakir et al., variable amounts of mixed aeroallergens may reach the lower airways, as a consequence of oral breathing secondary to nasal obstruction caused by allergic rhinitis. Our study design and characterization of allergen exposure were different from those of Chakir et al. We chose a cross-over protocol to compare the effects of nasal placebo or allergen challenge on bronchial airway responsiveness and inflammation in the same subject. The nature and the dose of allergen were controlled and always administered by standardized nasal challenge. Allergen inhalation cannot be excluded during nasal provocation. However,

studies with a radiolabeled allergen have not shown deposition of the allergen in the lungs after nasal allergen challenge performed with similar conditions as in our study (24). Moreover, as described in Material and methods section, careful precautions were taken to avoid swallowing or inhalation of allergen and nasal secretions during nasal provocation by our patients.

At baseline, our study population had significant, although low percentage of eosinophils, in induced sputum [2.9 (0–30.8)%]. One of our patients had more than 25% of eosinophils. In agreement with other studies, nonasthmatic patients with seasonal allergic rhinitis have frequently high number of eosinophils in their induced sputum or bronchial biopsies outside the pollen season (9, 10, 22). High eosinophils in baseline sputum may also be due to more extensive atopic sensitization than grass pollen only, especially in three of our patients.

In our study, no change in pulmonary function was detected early (H1) and late (H6) after nasal allergen challenge (data not shown). Similarly, Corren et al. did not find any significant change in pulmonary function 30 min and 4½ h after nasal allergen challenge in a cross-over study in seasonal allergic rhinitis patients with asthma symptoms (24). However, Braunstahl et al. (13) have reported reduced peak expiratory flows 4 and 6 h after nasal allergen challenge in seasonal allergic rhinitis patients without asthma as compared with control subjects. More recently a study showed an impairment of spirometric parameters in patients with seasonal allergic rhinitis and without asthma during the pollen season (19).

Two authors used quite similar study design as ours, but bronchial inflammation was evaluated later (24 h after nasal allergen challenge) and bronchial responsiveness to methacholine was not assessed in parallel (13, 20). It should be noted that Corren et al. (24) have shown that experimentally induced nasal dysfunction by allergic challenge resulted in increased bronchial hyperresponsiveness to methacholine, but they did not evaluate bronchial inflammation in parallel. Braunstahl et al. found increased number of eosinophils in the bronchial epithelium ($P = 0.05$) and subepithelium ($P = 0.02$) of patients with allergic rhinitis 24 h after nasal allergen challenge as compared with control subjects (13). Beeh et al. did not find any significant variation in eosinophil number 24 h after nasal allergen challenge in patients with allergic rhinitis and without bronchial hyperresponsiveness to methacholine outside the pollen season (20). Although IL-5 was undetectable and no increase of inflammatory cells was found in induced sputum of their patients, Beeh et al. reported that plasmatic levels of IL-5 increased after allergen challenge.

In our study, the increase in eosinophils number in induced sputum after allergic nasal challenge compared to placebo was associated with increased eosinophils expressing eosinophil-cationic protein (EG2+ eosinophils, $P = 0.03$) and eosinophil-cationic protein concentration in sputum supernatant ($P = 0.06$). Inverse

correlations between eosinophil number, eosinophil-cationic protein concentration and methacholine PD20 were observed after nasal challenge ($r = -0.57$, $P = 0.04$, $n = 12$ and $r = -0.80$, $P = 0.003$, $n = 12$ respectively). The relationship between eosinophils and methacholine PD20 supports the hypothesis that mediator release by activated eosinophils contributes to changes in airway responsiveness after nasal allergen challenge in patients with allergic rhinitis as previously reported for asthmatic subjects after bronchial allergen challenge (26).

Chemokines and cytokines involved in eosinophil recruitment and activation which were evaluated in induced sputum in this study were eotaxin-1, IL-5 and GM-CSF. Eotaxin-1 and GM-CSF were probably produced at too low levels to be detected by ELISA in sputum supernatant of our patients. Interestingly, IL-5 was slightly increased in sputum supernatant of three of our patients after nasal allergen challenge compared with placebo. One mechanism that could explain the interaction between eosinophilic inflammation in the nose and the lower airways in patients with allergic rhinitis is a bronchial aspiration of nasal inflammatory cells or mediators. Supporting this hypothesis is the effect of nasal corticosteroids on bronchial hyperresponsiveness in patients with allergic rhinitis (33). The possibility of a contamination of bronchial airways with nasal secretion through aspiration cannot be excluded with the present study. However, in our patients, 2 h before induced sputum was performed (H5–H7), no nasal inflammatory cells or mediators were found in nasal lavage fluids. Nasal blockage index and nasal symptom score were back to control levels at H5 when nasal lavage was performed. This may explain why no nasal inflammatory cell was recovered in lavage. Nevertheless, contamination of bronchial airways with nasal secretion through aspiration may have occurred earlier, from the end of nasal challenge procedure to the time of nasal lavage (H0–H5).

The existence of a systemic cross-talk between nose and lungs constitutes the second mechanism which could explain bronchial eosinophilic inflammation in our patients. Systemic IL-5 may contribute to local eosinophil accumulation (2, 7). Unfortunately, we did not measure plasma levels of IL-5 during the 6 h after nasal challenge. Beeh et al. found that these levels were increased 24 h after nasal allergic challenge but have not evaluated IL-5 concentration earlier (20). As systemic parameters were not measured in our study and nasal inflammation was quite low, a nasobronchial reflex cannot be excluded. Although this reflex has been well documented in animals, conflicting results have been reported in human studies (2).

As observed by Beeh et al. (20), we found that IL-10 levels were decreased after nasal allergic challenge compared with placebo ($P = 0.06$). This corroborates the results of Gauvreau et al. who found a decrease of IL-10 positive cells after bronchial allergen challenge in asthmatic patients (26). IL-10 is known to inhibit the

production of proinflammatory cytokines and chemokines by many cell types and particularly eosinophils (34). Through downregulation of this production, IL-10 may regulate recruitment and activation of eosinophils. Interestingly, macrophages, which are known to produce IL-10, were decreased after nasal allergic challenge in our study as previously described in asthmatic patients after bronchial allergen challenge (26).

Conclusions

We found that airway responsiveness was increased after nasal challenge with grass pollens and was not associated with changes in nasal symptoms and cellularity in patients with seasonal allergic rhinitis but without asthma outside the pollen season. Nasal allergic challenge induced increase in eosinophil number and

EG2+ eosinophils in induced sputum. This eosinophilic bronchial inflammation was correlated to airway responsiveness.

Our results confirm that eosinophils participate to the bronchial inflammation in nonasthmatic patients with seasonal allergic rhinitis following allergic nasal challenge outside the pollen season, which might explain changes in airway responsiveness. The bronchial eosinophilic inflammation could be explained by a production of nasal and/or systemic eosinophilic chemotactic factors. Further studies are needed to explore these mechanisms.

Acknowledgments

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