

Original article

Airway neutrophil inflammation in nonasthmatic patients with food allergy

Background: Patients with food allergy (FA) have been recently shown to develop bronchial hyperresponsiveness (BHR), despite the absence of any concomitant asthmatic manifestation. In order to explain this observation, we sought to examine the presence of a bronchial inflammation in induced sputum of nonasthmatic patients with FA.

Methods: Twelve nonasthmatic patients with FA (urticaria, digestive symptoms, anaphylaxis) were included in the study. Results were compared to those obtained from eight asthmatic patients without food allergy and eight healthy controls. Diagnosis of FA was based on double-blind placebo-controlled challenge. Sputum cells and fluid-phase eosinophil cationic protein (ECP), myeloperoxidase (MPO) and interleukin-8 (IL-8) were measured in induced sputum. BHR was evaluated using methacholine inhalation.

Results: Sputum from asthmatics, in comparison with the sputum of healthy subjects and patients with FA contained a higher proportion of eosinophils and higher levels of ECP ($P < 0.001$). In marked contrast, patients with FA exhibited an increased proportion of neutrophils and IL-8 in comparison with asthmatics and controls ($P < 0.05$ for neutrophils and $P < 0.001$ for IL-8). There was a significant correlation between sputum neutrophils and IL-8 ($r = 0.68$, $P < 0.001$). MPO levels were not different between the groups. There was a trend toward higher levels of IL-8 and ECP in food allergic patients with BHR in comparison with patients with FA without BHR.

Conclusion: Our results demonstrate that a subclinical neutrophil airway inflammation is present in patients with food allergy free of clinical respiratory symptoms and that IL-8 may be an important mediator of this neutrophilia.

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Subclinical inflammatory changes of bronchial mucosa have been recently demonstrated in patients with inflammatory bowel disease suggesting that a diffuse involvement of the mucosal immune system may occur in T-lymphocyte mediated inflammatory diseases (1). Clinical studies have also demonstrated an increased frequency of asymptomatic bronchial hyperresponsiveness (BHR) in nonasthmatic patients with inflammatory bowel disease like Crohn's disease or ulcerative colitis (2). These findings suggest that the inflammatory bowel disease by itself may favour the development of nonspecific BHR, probably through the activation of the common mucosal immune system (3). Along the same lines we reported that a subclinical BHR was present in nonasthmatic patients with food allergy (FA) (4). With this in mind, we have sought evidence for

airway inflammation in nonasthmatic patients with FA. To demonstrate this we used the method of sputum induction to study 12 consecutive nonasthmatic patients with FA, eight asthmatic patients without FA and a group of healthy controls matched for atopy.

Material and methods

Patients

Twenty-eight nonsmoking patients were included in the study. There were 12 nonasthmatic patients with FA but without food-induced asthma and without rhinitis, and eight untreated asthmatic patients without food allergy (Table 1). Eight healthy subjects without respiratory symptoms and without food allergy were studied as a control group. Informed written consent was obtained from all subjects and the study protocol was approved by the institutional review board (CP 98/102).

The diagnosis of FA was established using clinical criteria and selected laboratory procedures (5–8) including: 1) historical documentation of reactions to specific foods; 2) positive skin prick tests to specific food allergen and serum specific IgE antibodies

Abbreviations: ECP: eosinophil cationic protein; IL: interleukin; BHR: bronchial hyperresponsiveness; FA: food allergy.

Table 1. Clinical characteristics of the patients

	Controls (n=8)	Asthmatics without food allergy (n=8)	Nonasthmatic patients with food allergy (n=12)
Age (years)	33.1 ± 1.8	32.2 ± 5.9	32.3 ± 4.2
Urticaria	0	0	7
Angioedema	0	0	4
Anaphylactic shock	0	0	1
Digestive symptoms	0	0	2
Positive skin tests to pneumallergens	5 (62%)	5 (62%)	8 (66%)
House dust mite	5	5	6
Animal danders	0	0	1
Grass Pollens	1	1	5
Moulds	0	0	3
Eosinophils (/mm ³)	124 ± 26	258 ± 36*	453 ± 171*
Neutrophils (/mm ³)	5240 ± 271	5863 ± 239	5668 ± 228
Total IgE (KU/l)	94 ± 26	449 ± 144.4*	433 ± 177*
Food hypersensitivity			
Umbelliferous/drupaceous	0	0	9
Exotic fruits	0	0	2
Solanaceous	0	0	3
Peanut	0	0	3
Crustaceans	0	0	2

*Significantly different from controls ($P < 0.05$).
Results are expressed as mean ± SEM.

directed against the food allergen measured by the Phadebas radioallergosorbent test (RAST, Pharmacia Diagnostics Uppsala, Sweden); 3) resolution of symptoms under a strict food allergen free diet to observe whether the reported symptoms will resolve by removal of one or more foods from the diet; 4) rapid and reproducible clinical symptoms after a double-blind placebo controlled challenge; and 5) correlation between the positive food allergen ingestion challenge and the results of skin tests and serum specific IgE antibodies directed against the food allergen.

Asthma was defined according to the criteria suggested by the American Thoracic Society (ATS) (9). All asthmatic patients exhibited a history of episodes of dyspnoea and wheezes, and had a reversible airway obstruction characterized by a 20% increase in FEV₁ after the inhalation of 200 µg of salbutamol.

Respiratory evaluation included skin prick tests to aeroallergens (house dust mite, animal danders, pollens, moulds) and to trophallergens, spirometric measurements, methacholine challenge and induced sputum. Total IgE (Phadebas, paper radioimmunosorbent test; Pharmacia Diagnostics, Fairfield, NJ) as well as specific IgE levels (Phadebas radioallergosorbent test, Pharmacia Diagnostics) were measured. In patients with food allergy, respiratory evaluation was performed at least two months after diagnosis of FA and the last manifestations of FA. Exclusion criteria were FEV₁ < 70% predicted value, lower respiratory tract infection in the previous eight weeks, asthmatic exacerbation in the preceding eight weeks, and treatment with inhaled or oral corticosteroids in the four months before the study.

Prick tests

Skin prick tests were performed as recommended (10). Positive controls were histamine (10 mg/ml) and codeine phosphate. Wheal diameters with these preparations ranged from 3 to 7 mm. The negative control solution was the diluent used to preserve the allergen extracts. Skin tests were read at the peak of their reaction by measuring the wheal and the flare 15 min after the performance of the tests. Skin tests greater than 3 mm than the negative control were considered positive.

Food challenge

Double-blind food challenge (DBFC) was performed using apple compote as vehicle. The vehicle itself was used as placebo by giving a volume about the same as that containing the challenge. Prior to the challenge, β-agonists, theophylline and cromolyn were avoided for 48 h prior to the challenge. The DBFCs used one food or placebo per week. Food was prepared by a professional so that neither the patient nor the observer knew what the patient was receiving. An initial dose of 10–500 mg of dried food in the vehicle was administered. If no symptoms developed within 30 min the dose was doubled. Again the patient was observed for 30 min and increments progressed by doubling for several steps. The total dose to be administered approximated one serving of the food. Sequential evaluation of pulmonary function (FEV₁) was performed during food challenge (every 15 min during the first hour and then 15 min after each dose) and did not demonstrate significant changes. If no reaction was seen during the observation period, the challenge was judged negative (5, 6). As summarized in Table 1, all patients with food allergy exhibited type I hypersensitivity symptoms which occurred minutes or a few hours after ingestion of the incriminated food. The double-blind placebo controlled challenge was not performed in one patient who reported anaphylactic reactions after ingestion of the incriminated food (in this patient, the diagnosis of FA relied only on the criteria 1–3).

Pulmonary function tests and methacholine challenge

All measurements were made in the sitting position. FEV₁, FVC and maximal mid-expiratory flow (FEF₂₅₋₇₅) were obtained from flow-volume curves using a Medgraphics spirometer (St. Paul, MN). The largest values of FVC, FEV₁ and FEF₂₅₋₇₅ from the first three technically satisfactory forced expirations were selected. All data were expressed in absolute values and in percent of predicted normal values (11).

Methacholine challenge was performed sequentially with a DeVilbiss 646 nebulizer (output of 0.13 ml/min) (12). Patients inhaled for 2 min by tidal breathing first the diluent solution, followed by methacholine with doubling of concentration at 5-min intervals, starting from 0.125, to 16 mg/ml. The procedure was ended

when a 20% fall in FEV₁ from post-diluent value was obtained or when a methacholine concentration of 16 mg/ml was reached. The lowest, technically satisfactory FEV₁ value obtained between 90 s and 3 min was used in the analysis to calculate the dose-response curve. Dose-response curves were constructed by plotting FEV₁ against increasing concentration of methacholine as a log scale. The provocative concentration (PC₂₀) was defined as the methacholine concentration necessary to decrease FEV₁ by 20% from baseline values. BHR was defined by a PC₂₀ < 16 mg/ml. Asymptomatic bronchial hyperresponsiveness (BHR) was defined as a methacholine PC₂₀ < 16 mg/ml in the absence of symptoms suggestive of asthma (intermittent wheeze, chest tightness, dyspnoea, recurrent cough) in subjects who reported that they had never been told by a physician that they had asthma and never required any asthma medication. In patients with positive skin tests to seasonal aeroallergens, methacholine challenge was performed at least two months after the pollen season. Methacholine challenge was performed 1 h before sputum induction; previous reports have demonstrated that methacholine inhalation does not significantly alter the airway cellularity in healthy and in asthmatic subjects (13).

Sputum induction

Sputum was induced by the method described by Pizzichini and co-workers (14) by inhaling increasing concentrations of hypertonic saline (3%, 4% and 5%), each for 7 min, generated by a Devilbiss nebulizer (output 0.6 ml/min) through a mouthpiece without a valve or nose-clip. After each period of inhalation an FEV₁ was measured for safety. At the start and after each period of inhalation, the patients rinse their mouth, and swallow the water to minimize contamination from postnasal drip and saliva. Then they were instructed to cough sputum into a sterile container.

Sputum examination

The sputum was immediately examined: opaque or dense portions that looked different from saliva or were free of squamous cell contamination under the inverted microscope were selected from the expectorate, weighed and treated with 1/10 dilution of Sputolysin (Calbiochem Corp., San Diego, CA) in water (8.1 mg/ml of dithiothreitol) and phosphate-buffered saline (PBS) four times the weight of plugs (15). The resulting suspension was filtered and a total nonsquamous cell count was performed in a hemocytometer and expressed as millions per gram 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 filtrate, two cytopsins were made and stained with May-Grunwald Giemsa solution and a 400 differential nonsquamous cell count was performed. Another two cytopsins were stained with toluidine blue and 1500 cells were counted for metachromatic cells. The differential cell count, including metachromatic cells, was expressed as the absolute number of cells in millions per gram of sputum and as the percentage of the total nonsquamous cells. The specimen was considered adequate if total and differential cell counts could be obtained.

Fluid-phase measurements

The concentration of eosinophil cationic protein (ECP, sensitivity: 2 ng/ml) in the thawed supernatant was determined using a sensitive radioimmunoassay (RIA; Kabi Pharmacia Diagnosis AB, Uppsala, Sweden); myeloperoxidase (MPO, sensitivity: 1.5 ng/ml) was measured using sandwich ELISA (Oxys International, Inc., Portland, OR). Interleukin-8 (IL-8, sensitivity: 10 pg/ml) was measured by quantitative sandwich enzyme immunoassay (Quantikine; R & D Systems, Inc., Minneapolis, MN). The results were adjusted for the dilution. All sputum measurements were performed in duplicate blind to the clinical details.

Statistical analysis

Results are expressed as median and interquartile (IQR) or as mean $\pm$ standard error (SEM) when applicable. Most of the data were not normally distributed. For these data, statistical analysis was performed using the nonparametric Kruskal-Wallis test for differences among the groups. When this test indicated significant difference, each pairing was examined by means of the Mann-Whitney *U*-test. For normally distributed data (FEV₁%, FVC %, FEV₁/FVC and FEF₂₅₋₇₅%), an ANOVA test was applied to examine differences between the groups. When this test indicated significant difference, each pairing was examined by the Students *t*-test. Differences in qualitative data among the groups were examined by Fisher's Exact test. The correlations were investigated using the Spearman's rank correlation coefficient test. A probability value of less than 0.05 was taken as statistically significant.

Results

Characteristics of the subjects are reported in Table 1. There were no significant differences in terms of age, positive skin tests to aeroallergens between the groups. Blood eosinophil count and total IgE levels were higher in patients than in controls; neutrophil count did not differ between the groups. Pulmonary function test results are shown in Table 2. Six out of 12 (50%) nonasthmatic patients with food allergy exhibited an asymptomatic BHR (4). PC₂₀ was significantly lower in asthmatic patients than in nonasthmatic patients with food allergy.

The samples of sputum analyzed had little contamination with saliva as indicated by the proportion of squamous epithelial cells which was less than 2%. Total cell count per gram of sputum did not differ significantly between the three groups. Results are shown in Table 3. Sputum from asthmatics, in comparison with the sputum of healthy subjects and patients with FA had a higher proportion and number of eosinophils ($P < 0.001$) and higher levels of ECP ($P < 0.001$). Patients with FA also differed from healthy controls in eosinophil percentage and number ($P < 0.02$) and ECP level ($P < 0.01$). In marked contrast an increased proportion and number of neutrophils ($P < 0.05$) and of IL-8 ($P < 0.001$) was observed in patients with FA when compared to asthmatics and healthy controls (Fig. 1). As a consequence of the increase percentage of neutrophils and eosinophils, the percentage of macrophages was significantly lowered in asthmatic patients without FA and in nonasthmatic patients with FA ($P < 0.05$). Although there was a trend toward an increased level of MPO in nonasthmatic patients with FA, MPO levels were not significantly different between the three groups.

There was a highly significant correlation between the number and percentage of sputum neutrophils and the concentration of IL-8 (respectively $P < 0.001$, $r = 0.68$ and $P = 0.01$, $r = 0.58$). There was no correlation between eosinophils and ECP and no correlation between neutrophils and MPO levels and between IL-8 and MPO.

Table 1. Functional characteristics of the patients

	Controls (n=8)	Asthmatics without food allergy (n=8)	Nonasthmatic patients with food allergy (n=12)
FEV ₁ (%)	101 ± 4	91.1 ± 7.1	99.5 ± 3.2
FVC (%)	102 ± 2	100.5 ± 4.3	99.6 ± 4
FEV ₁ /FVC (%)	80 ± 0.1	76.1 ± 0.1	85.8 ± 0.1
BHR	0/8	8/8	6/12
SD ₂₀ (mg/ml methacholine)	—	2 ± 1.9	3.35 ± 0.74*

Results are expressed as mean ± SEM.

*Significantly different from asthmatics ($P < 0.05$).

Although there was a trend toward increased levels of ECP and IL-8 in food allergic patients with BHR, cellular characteristics of sputum and fluid-phase measurements did not differ according to the presence of BHR. There was no significant correlation between fluid-phase measurements and PC₂₀ although there was a trend toward a negative correlation between ECP levels and PC₂₀ in patients with FA (correlation ECP-PC₂₀: $r = -0.41$, $P = 0.08$).

Discussion

Our study clearly demonstrated that a subclinical bronchial inflammation is present in nonasthmatic patients with food allergy and that the neutrophil is the prominent inflammatory cell in the airway secretions of these patients.

Confounding factors that might be responsible for airway neutrophilia in patients with food allergy have been carefully excluded (16–18). All patients were lifelong nonsmokers. None of them had a recent or intercurrent respiratory tract infection; none of them had occupational exposure which might have resulted

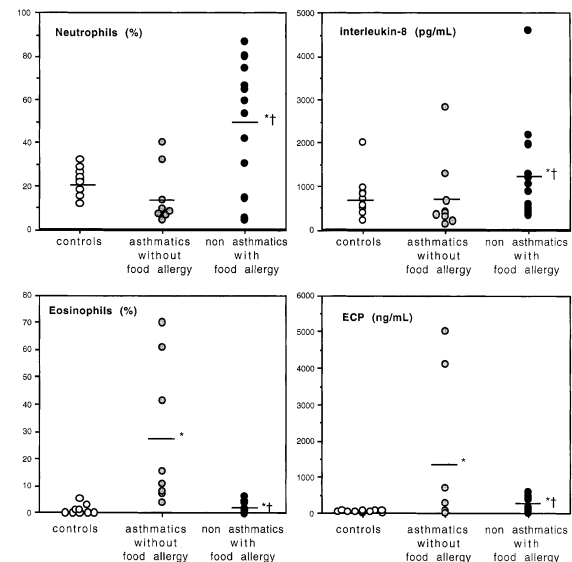


Figure 1. Sputum eosinophil and neutrophil percentage and fluid-phase ECP and IL-8 levels in healthy subjects, asthmatic patients without food allergy and nonasthmatic patients with food allergy.

in sputum neutrophilia. All subjects with FA were nonasthmatics (no previous history of asthmatic symptoms, normal pulmonary function and no FEV₁ changes during food challenge).

Although we found a high neutrophil count in induced sputum from patients with food allergy, fluid-phase MPO levels were not significantly increased and there was no correlation between neutrophil percentage and MPO levels. These results support the hypothesis that neutrophil recruitment in the airways is not associated with neutrophil activation. Interestingly airway neutrophil activation is usually associated with

Table 3. Total and differential cell count in induced sputum from controls, asthmatic patients and nonasthmatic patients with food allergy

		Controls	Asthmatics without food allergy	Nonasthmatics with food allergy
Induced sputum (mg)		36 (41)	68 (69)	38.5 (90)
Total cell count ($\times 10^6$)		0.29 (0.6)	0.58 (1.6)	0.34 (2.4)
Total cell count (10^6 cells/g sputum)	7.2 (6.4)	7.8 (33.8)	9.4 (14.8)	
Bronchial cells	%	3.6 (6.6)	9 (2.7)	4 (9)
	/g sputum	0.31 (0.35)	0.68 (2.1)	0.39 (0.92)
Macrophages	%	67 (5)	41 (39)*	30 (43)*
	/g sputum	5.1 (3.7)	1.9 (4.7)	2.8 (4.3)
Lymphocytes	%	4.1 (5.1)	2.7 (5.8)	2.5 (1.6)
	/g sputum	0.34 (0.34)	0.36 (1.3)	0.28 (0.42)
Eosinophils	%	0 (0.54)	13 (43)*	1.3 (3.5)*†
	/g sputum	0 (0.04)	3.2 (12)	0.2 (0.46)*†
Neutrophils	%	22.6 (9.2)	9.5 (15.3)	57 (47)*†
	/g sputum	1.4 (1.5)	0.8 (9.8)	6.2 (17)*†
Metachromatic cells	%	0 (0)	0 (0)	0 (0)
	/g sputum	0 (0)	0 (0)	0 (0)
MPO (ng/ml)		9.1 (22)	17.5 (35.6)	27.5 (40)

Results expressed as median (IQR).

*Significantly different from controls.

†Significantly different from asthmatics.

respiratory symptoms. For example, airway neutrophil recruitment and activation have been reported in patients with TDI-induced asthma following TDI bronchial challenge (19). Similarly increased neutrophils and cytokines associated with neutrophil activation have been reported in induced-sputum of nonasthmatic patients with chronic dry cough (20). The lack of neutrophil activation in patients with food allergy might explain, at least in part, why these patients showed airway neutrophil inflammation but did not exhibit clinical respiratory symptoms.

Eosinophilic inflammation of the airways is a recognized characteristic of asthma. Moderate bronchial eosinophilia and increased ECP levels were also found in sputum from nonasthmatic patients with FA. In our study there was a trend toward an increased percentage of eosinophils in food allergic patients with positive skin tests to aeroallergens, but the difference between patients with or without positive skin tests to aeroallergen did not reach statistical significance.

The clinical relevance of bronchial inflammation in food allergy is unclear. Interestingly, bronchial neutrophilia and eosinophilia in food allergy was not associated with the presence of BHR. Although BHR is often associated with bronchial eosinophilia in asthma (21), the association between BHR and sputum inflammatory indices remains controversial (22). Clearly BHR cannot be predicted by measuring sputum cellular or biochemical parameters (23). Polosa and co-workers suggested that methacholine responsiveness might predominantly depend on structural changes of the airways including airway wall remodelling and inability to dilate constricted airways (24). In our series there was no evidence of altered structure of the airways as judged by the absence of respiratory symptoms and the normality of pulmonary function values. Therefore the clinical relevance of bronchial neutrophilia and of moderate bronchial eosinophilia remains unclear. In addition respiratory follow-up of patients with food allergy and BHR over one year did not demonstrate occurrence of asthmatic symptoms although BHR persisted at the same level of PC₂₀ (4).

The mechanisms responsible for airway inflammation in patients with food allergy are likely multifactorial.

One can speculate that bronchial inflammation is a consequence of an allergic inflammatory process found in the gut mucosa of subjects with FA. We previously demonstrated that an airway-like inflammation occurs in gut mucosa and minor salivary glands of asthmatic patients suggesting that the whole mucosal system is involved as a cause or as a consequence in asthma (25–27). Conversely this study supports the concept that the inflammatory process of gut mucosa in FA may be responsible for a subclinical inflammation of the bronchi. It may be surprising to find an airway inflammation several months after the last clinical symptoms due to food allergy. However we previously reported that inflammatory processes of gut mucosa, including accumulation of eosinophils, lymphocytes and mast cells, persisted several months after the last clinical manifestation of FA (28). Strikingly inflammatory mucosal pattern clearly differs between gut and airways in FA. The reason why there is an accumulation of neutrophils in the airways of patients with FA is unclear. The accumulation of neutrophils is likely mediated by IL-8 because markedly elevated levels of IL-8 were found in airway secretions of patients with FA. In addition there was a positive correlation between IL-8 levels and neutrophils. It was beyond the scope of our study to identify the cellular source of IL-8 in the airways. Alveolar macrophages and/or airway epithelial cells are likely the cellular source of IL-8 since IL-8 was reported to be produced by these cells in several bronchial inflammatory diseases including asthma, cystic fibrosis, chronic bronchitis and bronchiectasis (29–32). One can hypothesize that circulating blood monocytes are activated in food allergy and may be responsible for IL-8 secretion at mucosal level (33).

In conclusion, our results demonstrate that a subclinical neutrophil airway inflammation is present in nonasthmatic patients with food allergy free of respiratory symptoms and that IL-8 is an important mediator of this neutrophilia. These results support the general concept that the whole mucosal immune system is involved in allergic diseases, even if the cell (s) or mediator (s) susceptible to make the link between the different mucosal systems are not yet elucidated (34).

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