NEUTROPHILIC CELLS IN SPUTUM OF ALLERGIC ASTHMATIC CHILDREN

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Airway inflammation is regarded as a central feature of asthma and is mostly sustained by eosinophilic infiltrate. Recent studies have shown that a co-activation of eosinophil- and neutrophil-dependent inflammatory mechanisms might explain why some asthmatics do not respond to conventional asthma therapy. The aim of our study is to determine whether neutrophilic inflammation was involved in 55 allergic children with mild-moderate persistent asthma and the relationship with the response to steroid treatment. Before the sputum analysis, all children underwent spirometry with the reversibility test, and were divided into two groups on the basis of the response (such as >12% of baseline FEV1): group 1 positive and group 2 negative. Eosinophil cationic protein concentrations were measured by radioimmunoassay and neutrophyl myeloperoxidase (MPO) concentrations were measured by an MPO-EIA. Ten healthy children of comparable ages served as control group. Total IgE, FEV1 and FEV/FVC values were similar in both groups. The sputum macrophage count was higher in controls than in allergic asthmatics, but there was no difference between groups 1 and 2 (59.6% *vs* 18.3% and 17%; p≤ 0.005). Sputum neutrophils were significantly higher in group 2 both *vs* controls (62% *vs* 34%; p≤ 0.005) and *vs* group 1 (62% vs 37%; p≤ 0.005). Our data suggest that neutrophils are involved in airway allergic inflammation in mild-moderate persistent childhood asthma and a high neutrophil count in sputum may be related to a lower responsiveness to inhaled corticosteroids.

Airway inflammation is considered a central feature of asthma and is mostly sustained by eosinophilic cells which infiltrate bronchial tissue (1). However, there is evidence that not all asthma cases are attributable to allergic inflammation, and other mechanisms may be involved in pathogenesis of the disease, including neutrophilic infiltrate (2). Furthermore, non-eosinophilic asthma is associated with neutrophilic responses not only in severe asthmatics (3-4), but also in individuals with moderate and mild asthma (5-7). Co-activation of eosinophil and neutrophil inflammatory mechanisms, observed in some cases of acute asthma (8), might explain why some asthmatics do not respond to conventional asthma therapy (2). Recent studies showed that airway inflammation in asthma could be categorized in different inflammatory subtypes based on the percentage of eosinophils and neutrophils infiltrating the bronchial wall (9). Thus, it has been reported that neutrophilic inflammation could be resistant

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to corticosteroid treatment, whereas eosinophilic inflammation could be sensitive to steroids (3, 10).

The aim of our study is to investigate how neutrophilic inflammation is involved in allergic airway inflammation. To this aim we measured inflammatory cells and mediators in induced sputum of allergic children with mild-moderate persistent asthma previously treated with inhaled steroids. In addition, the relationship between the inflammatory pattern and the response to steroid treatment was investigated.

MATERIALS AND METHODS

Patients and study design

Seventy-six allergic children, aged 6-10 years, with mild-moderate persistent bronchial asthma were progressively selected (1). All patients were well-matched for age, severity of asthma, and previous corticosteroids dosages. None of them had respiratory infections in the month before the study and all were clinically well-controlled after three months of inhaled steroid therapy (budesonide 200 mcg/b.i.d.), which was stopped two weeks before the sputum analysis. Before the sputum analysis, all the children were submitted to spirometry, including the reversibility test (see Methods below). A satisfactory sputum analysis and spirometry performance were not possible for 21 children; therefore, 55 children (35 males) were enrolled in the study. The children were divided into two groups on the basis of the response to reversibility test: an increase in baseline FEV1 values ≤ 12%, such as a negative response, characterized group 1 (34 children), whereas an increase over 12% characterized group 2 (21 children). Atopy was assessed by the skin prick test and RAST positivity (Pharmacia, Sweden) for the most common allergens. All children were sensitized (II-III classes) to house-dust mite and/or grass pollen. Ten non-atopic, non-asthmatic children of comparable ages served as control group.

Spirometry and reversibility tests

A dry spirometer (Sensor Medics, Vmax 22) was used for spirometry, conducted according to American Thoracic Society guidelines, and the best FEV1 of three manoeuvres was recorded and expressed as a percentage of predicted values. No child had used β2 agonist in the previous 24 hours. The reversibility test requires a FEV1 increase of more than 12% compared with baseline values, 15 min after inhalation of 200 μg albuterol delivered by metered dose inhaler.

Sputum induction and processing

Sputum was induced using the method described by Iredale et al. (7). The children inhaled 3.5% saline solution at room temperature, nebulized by an ultrasonic nebulizer at maximal saline output (4 ml). The total period of sputum induction was 15 min. The initial sample from the first cough was discharged. Sputum was kept at 4°C and processed within 2 h. The sample was homogenized with a balanced salt solution containing 1% dithiothreitol (DTT). The final concentration of DTT in all samples was 0.2%. The mixture was centrifuged at 400 g for 10 min at 4°C. Sputum supernatants were kept at –70°C for mediator assays. The cell pellets were re-suspended, and total cell counts were performed with a haemocytometer using Kimura stain. Slides were prepared with cytospin and stained with Papanicolau for differential cell count, which was performed by an expert observer, blind to the clinical characteristics of the subjects. At least 500 inflammatory cells were counted in each sample. A sample was considered adequate if it contained less than 50% of squamous epithelial cells on cytospin.

Mediator assays

Eosinophyl cationic protein (ECP) concentrations were measured by radioimmunoassay (Pharmacia, Uppsala, Sweden). The detection limit of the assay was <2 ng/ml. Neutrophil myeloperoxidase (MPO) concentrations were measured by an MPO-EIA (Oxis International, Inc., Portland, OR, USA) according to the manufacturer's instructions. The detection limit of assay was <1.6 ng/ml

Statistical analysis

Data are expressed as median (25°-75° percentile). The Wilcoxon test was used to compare the groups. Analysis of correlation was conducted with the Spearman rank correlation test and a p value less than 0.05 was considered significant.

RESULTS

Total IgE values, FEV1 and FEV/FVC were similar in groups 1 and 2 (Table I). However, there was a significant difference concerning the response degree to the bronchodilation testing ($p<.005$).

As shown in Fig. 1, the sputum macrophage count was higher in the control group than in allergic asthmatics, but there was no difference between the two patient groups (60% *vs* 18% and 18%; $p<0.005$). Sputum eosinophil and lymphocyte counts were significantly higher in asthmatic children than in controls (eosinophils: 13% *vs* 4% *vs* 0.05% $p\leq 0.005$; lymphocytes: 4% *vs* 3% *vs* 0% $p\leq 0.005$) but not significantly in group 1 versus group 2. Sputum

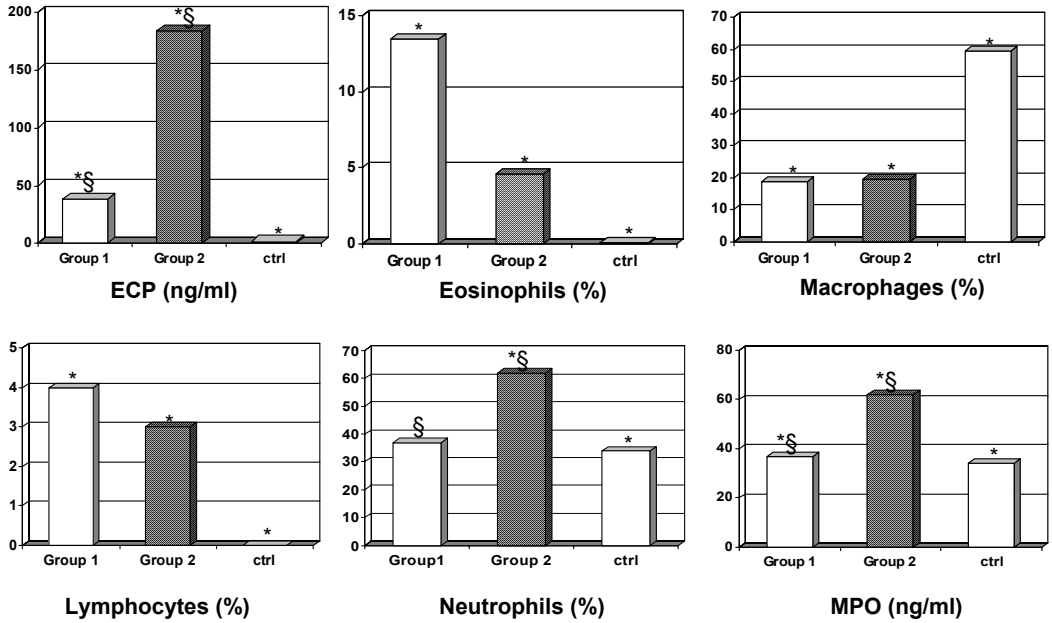

Fig. 1. *Sputum characteristics: ECP levels, eosinophil counts, macrophage counts, lymphocyte counts, neutrophil counts, and MPO levels in Group 1 (children with), Group 2 (children without reversibility to bronchodilation test) and controls (healthy children).*

neutrophils were significantly higher both in group 2 asthmatics versus group 1 and versus controls (62% *vs* 37% *vs* 34%; p≤ 0.005). Both ECP and MPO levels in induced sputa were significantly higher in the asthmatic children than in controls, but the levels were significantly higher in group 2 versus group 1 asthmatics (ECP: 191 *vs* 38.4; p≤ 0.005. MPO: 19.3 *vs* 4.6; p≤ 0.005). There was a significant correlation between ECP and MPO only in group 2 (Table II); similarly, there was a correlation between airway reversibility and ECP or MPO only in group 2 (Table II). There was no correlation between ECP and the proportion of sputum inflammatory cells in group 1. On the contrary, ECP levels correlated with the proportion of neutrophils in group 2. As expected, the proportion of neutrophils correlated with MPO levels in both groups of asthmatics (Table II).

DISCUSSION

This study suggests that neutrophils may be involved in airway inflammation in children with allergic asthma. The pathogenic mechanism of neutrophil recruitment in the airways is not well understood. It has not been clarified whether neutrophils are the main part of the inflammatory process or whether they may be secondary to high doses of inhaled corticosteroids (12), which are able to prolong neutrophil survival (13) and reduce eosinophil survival thus inhibiting apoptosis (14). Innate immune activation seems to be an important pro-inflammatory mechanism in neutrophilic asthma (15). Some authors reported that subjects with neutrophilic asthma were older, had a later onset of disease, and were less allergic than subjects with normal levels of neutrophils (16). Several inflammatory markers have been thought to be associated with non-eosinophilic inflammation in asthma (17). The most widely investigated is IL-8, a potent neutrophil chemoattractant and activator, which seems to be correlated with the sputum neutrophil counts in asthmatic patients (12, 18).

In our study, the presence of neutrophils in sputa was not a consequence of inhaled corticosteroid treatment. Probably, non-allergic neutrophilic asthma, which is poorly responsive to inhaled

Table I. *Characteristics of patients.*

	Group 1	Group 2	
Patients n.	34	21	
Males n.	21	14	
Total IgE (U/l)	300 (122-460)	260 (181-615)	
FEV1 %predicted	96 (91-105)	89 (83-100)	
FEV/FVC	95 (92-104)	91 (88-93)	
Airway reversibility %	**5 (2-8)**	**15 (15-20)**	**P<.005**

Values are expressed as median (25°-75° percentile)

Table II. *Correlations between mediators and cell types in sputum.*

	Group 1	Group 2
	Z corrected for ties	Z corrected for ties
ECP *vs* MPO	1.136	**2.621***
ECP *vs* airway reversibility	- 0.292	**- 2.236***
MPO *vs* airway reversibility	- 1.23	**- 3.092***
ECP *vs* eosinophils%	1.048	1.034
ECP *vs* macrophages %	- 0.059	-0188
ECP *vs* neutrophils %	- 0.157	**2. 297***
ECP *vs* lymphocytes%	-0.394	1.477
MPO *vs* eosinophils%	- 0.97	0.369
MPO *vs* macrophages %	- 1.963	1.406
MPO *vs* neutrophils %	**2.651***	**3.031***
MPO *vs* lymphocytes%	- 0. 383	1.896

** p≤.005*

corticosteroids, is another different phenotype setting (in terms of cell counts and response to steroids) (19).

We found no significant statistical difference between group 1 and group 2 in the FEV1 and FEV1/FVC percentage predicted. Moreover, we found no correlation between sputum eosinophil and neutrophil count and FEV1 values, in accordance with other authors (16).

These functional and cytological findings may indicate that group 2 children have a neutrophilic inflammation and negative reversibility test as a possible consequence of a poor response to inhaled corticosteroids. This finding is consistent with a study of Pavord et al. (20) who found that non-eosinophilic asthma is associated with a poor response to inhaled steroid, suggesting that sputum eosinophil count is an important factor in determining the response to inhaled steroid in asthma.

There was also a significant correlation between sputum ECP and MPO levels and between ECP levels and neutrophil counts in group 2 children. As suggested by others (21), the increase in the number of bronchial neutrophils, ECP and MPO may be mediated by increased levels of the potent chemokine IL-8. In fact, Gibson et al. (18) showed that levels of IL-8 correlated with ECP and MPO in sputum super-natants, which is consistent with a role for IL-8 as a degranulating agent for both eosinophils and neutrophils.

Very interestingly, high levels of sputum ECP were observed in non-eosinophilic asthma. However, increased ECP has been seen in the absence of eosinophils in other neutrophil-mediated airway disease, such as bronchiectasias (22), suggesting that neutrophils may contain ECP (23-24). Furthermore, neutrophils from atopic asthmatics have an increased propensity to release MPO, which may be modified by immunotherapy (25).

Why neutrophilic inflammation determines a poor response to corticosteroid therapy is not completely known; most likely other inflammatory processes occur in the airways. However, neutrophil products

could cause airway narrowing, increased mucus secretion (26), and increased airway smooth-muscle responsiveness (27). More in-depth investigation into inflammatory processes and effective treatment strategies in neutrophilic inflammation in asthma are certainly required.

In conclusion, our results suggest that neutrophilic infiltrate could represent a primary pathological process in asthmatic inflammation and that the neutrophilic share of bronchial inflammation may be related to a reduced responsiveness to inhaled corticosteroids in allergic asthmatic children.

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