

Markers of eosinophilic and neutrophilic inflammation in bronchoalveolar lavage of asthmatic and atopic children

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Abstract

Background: Recent studies performing fiberoptic bronchoscopy in children have improved our understanding of asthma pathophysiology. Eosinophilic, but also neutrophilic, inflammation has been described in asthma, but the relationship with atopy was incompletely investigated. The aim of this study is to examine inflammatory cells and mediators in children with asthma compared to the appropriate controls, i.e. atopic children without asthma and children with no atopy or asthma. Moreover, asthmatic children were analysed separately based on the presence of atopy and stratified by age.

Methods: We recruited 191 children undergoing fiberoptic bronchoscopy for appropriate indications: 91 asthmatics (aged 1.4–17 years), 44 atopics without asthma (1.6–17.8 years) and 56 nonasthmatic nonatopic controls (1.4–14 years). In bronchoalveolar lavage, total and differential cell counts and inflammatory mediators, including ECP, eotaxin, IL-8 and TNF α , were analysed.

Results: Eosinophils and ECP levels were increased in asthmatic children when compared to controls ($P = 0.002$ and $P = 0.01$, respectively), but also atopic children without asthma had increased ECP levels compared to controls ($P = 0.0001$). Among asthmatic children, eosinophils and ECP levels were not different between atopic and nonatopic individuals. Neither neutrophils nor the related mediators (IL-8 and TNF α) differed significantly in the three groups. This pattern of inflammation was observed in both preschool and school-aged asthmatic children.

Conclusions: This study suggests that markers of eosinophilic, but not neutrophilic inflammation, are increased in asthmatic children and also in atopic children without asthma. Of interest, in asthmatic children, the activation of the eosinophilic response is not solely because of the presence of atopy.

Asthma is a chronic inflammatory disease of the lower airways in which many cells and cellular elements play a role. The air-

way inflammatory response involves eosinophils, mast cells and activated T lymphocytes (1, 2) and is associated with structural alterations of the bronchial wall. These changes consist of desquamation of bronchial epithelial cells, basement membrane thickening, angiogenesis and increased smooth muscle mass (3, 4). The molecular mechanisms responsible for the recruitment and activation of inflammatory cells are still incompletely understood, particularly in the first years of life.

Recently, flexible fiberoptic bronchoscopy has become an important tool in the investigation of infants and children with asthma. Bronchoalveolar lavage (BAL) is a routine part of the procedure useful not only for microbiological investi-

Abbreviations

BAL, bronchoalveolar lavage; BDR, bronchodilator reversibility; ECP, eosinophilic cationic protein; FB, fiberoptic bronchoscopy; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroids; IgE, immunoglobulin E; IL-8, interleukin 8; kDa, kiloDalton; MCP, monocyte chemotactic protein; OSAS, obstructive sleep apnea syndrome; PRIST, paper-radio-immunosorbent-test; RAST, radio-allergo-sorbent-test; SEM, standard error of the mean; TNF α , tumour necrosis factor α .

gation, but also to study cellular and noncellular factors associated with inflammation. Inflammatory cells and several mediators of inflammation have been detected in BAL of asthmatic children when compared to nonaffected ones. Indeed, increases in eosinophils, eosinophil cationic protein (ECP) and eosinophil-related cytokines (RANTES, MCP and eotaxin) have been described in several studies (5–8). Some reports also showed increased airway neutrophilic inflammation that correlated with expression of IL-8 (9–12).

However, in the majority of those studies, whether these inflammatory changes were related to the atopic status was not investigated. This issue is particularly relevant in children, because several phenotypes of wheezing are described in the literature (13, 14). In particular, while symptoms in nonatopic wheezers are thought to resolve by adolescence, children with atopy and wheeze have a considerably higher risk of symptoms persistence in adult life (15–19). At present, little is known about the characteristics of the inflammatory reaction in atopic and nonatopic asthmatic children, as well as in atopic children without asthma. While in a BAL study, Stevenson and et al. reported that eosinophil numbers were increased in atopic children with asthma when compared to atopic children without asthma (5), we demonstrated a similar tissue eosinophilia in atopic children either asthmatics or nonasthmatics (20, 21). Moreover, among asthmatics, some studies found increased eosinophilia in atopic vs nonatopic individuals (22, 23), while others did not report such a difference (24, 25).

The aim of this study was to examine cell counts and inflammatory mediators (ECP, eotaxin, IL-8 and TNF α) in a group of children with asthma compared to the appropriate controls of atopic children without asthma and children with no atopy and no asthma. Moreover, asthmatic children were analysed separately according to the presence or absence of atopy and stratified by age.

Methods

Subjects

We recruited three groups of subjects: 91 children with asthma, 44 atopic children without asthma and 56 nonatopic non-asthmatic children. All children underwent fiberoptic bronchoscopy (FB) for appropriate clinical indications (26, 27).

Children were defined as having asthma when they had repeated episodes of wheezing, breathlessness and cough, particularly at night and early morning, which were present even apart from colds. Moreover, in all asthmatic children, symptoms had to be responsive to bronchodilators. Presence and reversibility of episodic symptoms were assessed by parental reports and confirmed by the child's paediatrician. The reason for this choice was to have the same diagnostic criteria in all children, including those too young to perform spirometry (20).

The presence of atopy was defined by an increase in total (paper-radio-immuno-sorbent-test, PRIST) or specific (radio-allergo-sorbent-test, RAST) IgE. In particular, specific IgE for the following aeroallergens were investigated: house dust mite (*Dermatophagoides pteronyssinus*, *Dermatophagoides*

farinae), moulds (*Alternaria alternata*), cat dander and grass pollens (*Lolium perenne*, *Poa pratensis*, *Phleum pratense*, *Dactylis glomerata*, *Cynodon dactylon*). All assays for specific IgE were judged to be positive if they had in excess of 0.35 kU/l out of specific allergen (28). Furthermore, because this definition of atopy is permissive, all analyses were also performed using a more stringent definition that included as atopic only subjects with specific IgE > 0.35 kU/l.

Fiberoptic bronchoscopy with BAL was performed according to the ERS guidelines for FB in children (27, 29). Asthmatic children underwent FB for recurrent pneumonia ($n = 54$), chronic cough ($n = 19$), difficult asthma ($n = 13$), stridor ($n = 2$), middle lobe syndrome ($n = 2$) or obstructive sleep apnea syndrome (OSAS) ($n = 1$); atopic children without asthma for recurrent pneumonia ($n = 31$), stridor ($n = 2$), chronic cough ($n = 10$) or middle lobe syndrome ($n = 1$) and control children for recurrent pneumonia ($n = 29$), stridor ($n = 5$), chronic cough ($n = 21$) or laryngeal angiomatosis ($n = 1$). Children with clinical suspicion of bronchiectasis underwent CT scan and, when confirmed, were excluded from the study.

All children underwent routine blood tests, whereas spirometry was carried out only in children who were able to perform the test. FEV₁ was measured using a 10-L bell spirometer (Biomedin, Padua, Italy), and the best of three measurements was expressed as a percentage of predicted values (30). In asthmatic children with baseline FEV₁ less than 80% pred, measurements were repeated 15 min after the inhalation of 400 μ g salbutamol via a spacer. Bronchodilator reversibility (BDR) was expressed as per cent FEV₁ change compared to baseline value.

The study was approved by the Ethical Committee of our Institution, and written consent was obtained from children's parents.

FB and BAL

Fiberoptic bronchoscopy was performed as previously described (12). BAL was carried out using normal sterile saline solution warmed to body temperature (37°C). BAL volume was adjusted to body weight using 1 ml/kg divided into three equal fractions in children weighing < 20 kg and 3 ml/kg of normal saline in children weighing > 20 kg (29). The fluid was recovered by mechanical aspiration into a suction trap using pressure of 25–100 mmHg. The first sample collected was used for culture, and values > 10⁵ CFU/ml of BAL fluid were considered to indicate bacterial infection (12). The other two aliquots were pooled together and submitted for cytological studies and analysis of BAL components, including proteins and inflammatory mediators. BAL was considered technically acceptable if recovery was $\geq 40\%$.

Furthermore, in 59 out of the 191 children included in the study, bronchial biopsies were available. These have been evaluated to quantify the eosinophil infiltration in other studies and processed as previously described (20, 21, 24). The data have been used to investigate correlations between tissue eosinophilia and the parameters examined in the present work.

Cellular counts

Before assessing the cellular and noncellular component, the fluid was filtered through gauze and centrifuged at 500 *g* for 10 min. After eliminating the supernatant, the cell pellet was suspended in 1 ml of Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) and centrifuged (Phicom Co., Cheshire, UK), spinning = 1×10^6 cells/ml. The centrifuged cell preparations were air-dried and stained with May-Grumwald-Giemsa for the differential count of macrophages, lymphocytes, eosinophils and neutrophils. The total cell values and the different cell populations were expressed as $10^6/\text{ml}$ and in per cent of total cell count.

Mediators in BAL fluid

The quantitative evaluation of BAL ECP was performed using an immunofluorescence assay (ImmunoCAP 250 and ImmunoCAP 1000; Phadia, Milan, Italy). The lower limit of detection for ECP in BAL was 2 $\mu\text{g}/\text{l}$, and the upper limit was 200 $\mu\text{g}/\text{l}$.

IL-8 and TNF α analyses were performed using the Immulite assay (Immulite one; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The technique is based on a solid-phase two-site chemiluminescent enzyme immunometric assay. The lower limit of detection for IL-8 was 1 pg/ml, and the upper limit was 7500 pg/ml. The lower limit of detection for TNF α was 1.7 pg/ml, and the upper limit was 1000 pg/ml.

Eotaxin measurement was performed by a quantitative sandwich enzyme immuno-assay (R&D Systems, Minneapolis, MN, USA). Results were expressed in pg/ml, and minimum detectable dose is < 5 pg/ml.

Statistical analysis

Cell counts, ECP, eotaxin, IL-8 and TNF α values are expressed as median and range. Screening of differences in BAL mediators and cells was performed first using the Kruskal–Wallis test which, if significant, was followed by

between-two-groups comparisons using the Mann–Whitney test. Age, FEV₁ and FEV₁/FVC were expressed as mean $\pm$ SEM and were compared using the analysis of variance and unpaired Student *t*-test. The reported *P*-values are those of between-two-groups analyses adjusted for multiple comparisons using the Bonferroni/Holm correction. Correlations between values were assessed by calculating Spearman's correlation coefficients. A *P*-value < 0.05 was considered statistically significant.

Results

Clinical findings

One hundred and ninety-one children (89 females, 46.6%) were included in this study: 91 (47.6%) were asthmatics, 44 (23.1%) were atopic nonasthmatics and 56 (29.3%) were nonatopic nonasthmatics. The clinical characteristics of the children studied are given in Table 1. Spirometry was successfully performed in 52 out of 91 children with asthma, 24 out of 44 atopic nonasthmatic children and 21 out of 56 control children. FEV₁ values were significantly lower in asthmatic children than controls. In asthmatic children with baseline FEV₁ less than 80% predicted (*n* = 9), the mean response to bronchodilator was $23 \pm 7\%$ of baseline values. Among asthmatic children, there were 54 atopic and 37 nonatopic subjects, who were similar for age, FEV₁ and FEV₁/FVC values.

Fifty-five of 91 (60.4%) asthmatic children had mild asthma and were treated with inhaled salbutamol only when needed. The remaining 36 (39.6%) children had moderate or difficult asthma. Equivalent daily doses of beclomethasone ranged from 200 to 400 μg for children with moderate asthma and were ≥ 800 μg for children with difficult asthma. None of the children was currently treated with oral corticosteroids or antibiotics at the time of FB and BAL.

Pathogenic bacteria were cultured from 24 (26.4%) of the asthmatic children (10 *H. influenzae*, 12 *S. pneumoniae*, 1 *M. pneumoniae*, 1 *H. influenzae* plus *S. pneumoniae*); 13

Table 1 Clinical characteristics of children

	Asthmatic children	Atopic non asthmatic children	Control children
Number and sex (M/F)	91 (58/33)	44 (14/30)	56 (30/26)
Age (months)	83 $\pm$ 4	82 $\pm$ 7	66 $\pm$ 4
FEV ₁ (% predicted)*	90 $\pm$ 2†	94 $\pm$ 2‡	102 $\pm$ 2
FEV ₁ /FVC (%)	91 $\pm$ 2	95 $\pm$ 3	94 $\pm$ 2
BDR (Δ FEV ₁ % baseline)	23 $\pm$ 7§	–	–
Circulating eosinophils (cells/mm ³)	443 $\pm$ 41¶	395 $\pm$ 41‡	254 $\pm$ 37
IgE (U/ml)	391 $\pm$ 75†	280 $\pm$ 59†	23 $\pm$ 3

Values are expressed as mean $\pm$ SEM.

Definition of abbreviations: M = male; F = female.

*Pulmonary function testing was performed in 52 asthmatic, 24 atopic non asthmatic and 21 control children.

†*P* < 0.001 vs control children.

‡*P* < 0.05 vs control children.

§BDR was performed in 9 asthmatic children.

¶*P* < 0.005 vs control children.

(29.5%) of the atopic nonasthmatic children (8 *H. influenzae*, 2 *S. pneumoniae*, 3 *H. influenzae* plus *S. pneumoniae*) and 10 (17.2%) of the controls (3 *H. influenzae*, 4 *S. pneumoniae*, 2 *M. catarrhalis*, 1 *H. influenzae* plus *S. pneumoniae*).

Serum eosinophil counts and IgE levels were significantly increased in both asthmatic and atopic nonasthmatic children when compared to controls. Fiberoptic bronchoscopy and BAL were well tolerated by all children, and no major complications were observed.

BAL cells and mediators

Total, differential cells counts and mediator levels are summarized in Table 2. The total cell number was similar in the three groups of children examined.

Eosinophils, expressed both as absolute numbers and percentages, were increased in asthmatic children when

compared to control children ($P = 0.007$ and $P = 0.002$, respectively). The numbers and percentages of eosinophils in atopic nonasthmatic children were not significantly different from asthmatics, even if they were not increased when compared to controls (Fig. 1). Conversely, ECP levels were increased both in asthmatic and in atopic nonasthmatic children when compared to controls ($P = 0.01$ and $P = 0.0001$), with no differences between the asthmatic and atopic nonasthmatic children (Fig. 1). The levels of eotaxin did not differ significantly among the three groups of children examined.

Although an important neutrophilia was detected in all groups, no difference was observed in either neutrophil numbers or percentages among asthmatic, atopic nonasthmatic and control children. Similarly, the levels of IL-8 and TNF α did not differ significantly among the three groups of children examined (Table 2).

Table 2 Cellular counts and proteins in BAL

	Asthmatic children (91)	Atopic nonasthmatic children (44)	Control children (56)
Total cells ($\times 10^6/\text{ml}$)	0.167 (0.017–14.700)	0.190 (0.018–1.060)	0.250 (0.0017–2.025)
Eosinophils ($\times 10^6/\text{ml}$)	0 (0–1.176)*	0 (0–0.027)	0 (0–0.067)
Eosinophils (%)	0 (0–29)†	0 (0–14)	0 (0–9)
ECP ($\mu\text{g/l}$)	14.7 (2.0–200)‡	23.1 (2.6–200)§	7.8 (2.0–200)
Eotaxin (pg/ml)¶	13.0 (1.8–45.0)	14.8 (5.0–22.5)	15.0 (7.6–52.0)
Neutrophils ($\times 10^6/\text{ml}$)	0.019 (0–7.938)	0.024 (0–0.644)	0.034 (0–1.802)
Neutrophils (%)	10 (0–92)	17 (1–92)	17 (0–91)
IL-8 (pg/ml)¶	315 (35–7500)	590 (24–7500)	437 (5–5728)
TNF α (pg/ml)¶	9.0 (4.0–66.6)	10.8 (4.0–423)	8.5 (4–158)

Values are expressed as median (range).

* $P = 0.007$.

† $P = 0.002$ vs control children.

‡ $P = 0.01$ vs control children.

§ $P = 0.0001$ vs control children.

¶IL-8 was measured in 74 asthmatic, 31 atopic nonasthmatic and 46 control children; TNF α in 61 asthmatic, 27 atopic nonasthmatic and 40 control children; eotaxin in 25 asthmatic, 9 atopic nonasthmatic and 18 control children.

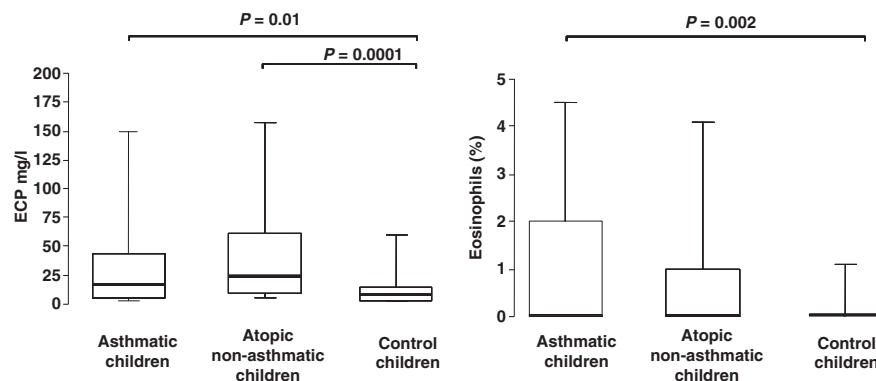


Figure 1 ECP levels (panel A) and eosinophil percentages (panel B) in asthmatic ($n = 91$), atopic nonasthmatic ($n = 44$) and control children ($n = 56$). In each box plot, the bottom and the top of the

box denote the 25th and 75th percentiles, respectively, the solid line is the median and the brackets are the 10th and 90th percentiles.

Atopic vs nonatopic asthmatics

When asthmatic children were subgrouped according to the presence of atopy, we found that eosinophil numbers, percentages and ECP were not different between atopic and nonatopic asthmatic individuals (Table 3). By contrast, eotaxin levels were lower in nonatopic asthmatic children when compared to atopic ones. Again, neutrophils and neutrophil-related mediators (IL-8 and TNF α) were not different between atopic and nonatopic asthmatic children (Table 3).

When the analysis was performed using a more stringent definition of atopy (requiring positive RAST, i.e. specific IgE > 0.35 kU/l), all results were confirmed. Indeed, among asthmatic children, BAL eosinophils and ECP were not different between those with positive RAST and those with negative RAST (0, 0–29% vs 0, 0–24% and 16, 2–200 μ g/l vs 12, 2–200 μ g/l). Similarly, BAL neutrophils, IL-8 and TNF α were not different between asthmatic children with positive and negative RAST (7, 0–92% vs 16, 0–77 and 265, 35–6238 pg/ml vs 337, 44–7500 pg/ml and 9.3, 4.0–66.6 pg/ml vs 9.0, 4.0–58.4 pg/ml). Finally, the levels of eotaxin were decreased in asthmatic children with negative RAST when compared with asthmatic children with positive RAST (11.0, 1.8–43.8 pg/ml vs 16.0, 3.2–45.0 pg/ml, $P = 0.01$), as found in the original analysis with the broad criteria for definition of atopy.

Age-stratified analysis

We then performed an age-stratified analysis considering separately children < 3 years of age, those aged between 3 and 6 years and those > 6 years.

Asthmatic children > 6 years ($n = 42$) had increased eosinophilia and ECP levels when compared to age-matched control children ($n = 15$) (0, 0–24% vs 0, 0–2%, $P = 0.04$

and 12.3, 2–200 μ g/l vs 5.2, 2–138 μ g/l, $P = 0.05$). These results were maintained in asthmatic children $3 \leq \text{aged} \leq 6$ - years ($n = 38$) when compared to controls of the same age ($n = 35$) (0, 0–12% vs 0, 0–22%, $P = 0.02$ and 18.8, 2–200 μ g/l vs 7.8, 2–200 μ g/l; $P = 0.01$). However, when we considered children < 3 years, the differences between asthmatics ($n = 10$) and controls ($n = 5$) were no longer evident (0, 0–2% vs 0, 0–4% and 12.2, 2–95 μ g/l and 13.9, 2–200 μ g/l). Neutrophil numbers and related mediators were not different between asthmatic children and age-matched control children at any time-point.

When we considered the influence of atopy among school-aged and preschool asthmatics, we found no differences between school-aged children with positive RAST and school-aged children with negative RAST (eosinophils: 0, 0–17% vs 0, 0–24% and ECP: 12.3, 2–200 μ g/l vs 12.4, 2–139 μ g/l) nor between preschool children with positive RAST and preschool children with negative RAST (eosinophils: 0, 0–12% vs 0, 0–7% and ECP: 29.1, 2–200 μ g/l vs 16.8, 2–200 μ g/l).

Potential confounders

To rule out the possibility that bacterial infection could have affected cell counts or mediators, we excluded children with positive BAL cultures from the analysis. The main results of our study, with regard to eosinophils and ECP, were confirmed. Indeed, asthmatic children with negative BAL cultures had increased eosinophilia and ECP levels when compared to control children with negative BAL cultures (0, 0–29% vs 0, 0–9%; $P = 0.009$ and 14, 2–200 μ g/l vs 5, 2–200 μ g/l; $P = 0.009$). Moreover, among asthmatics, atopic children with negative BAL cultures were not different from nonatopic children with negative BAL cultures as for eosinophils and ECP (0, 0–29% vs 0, 0–24% and 14, 2–200 μ g/l vs 9, 2–200 μ g/l), while nonatopic children had decreased levels of eotaxin (8.8, 1.8–43.8 pg/ml vs 17.0, 3.2–43.8 pg/ml, $P = 0.01$).

When we compared, among asthmatic children, those with positive to those with negative BAL cultures, no differences were observed for eosinophils and related mediators, but children with positive cultures had increased neutrophils and increased TNF α (21, 1–92% vs 9, 0–74%; $P = 0.01$ and 12, 4–67 pg/ml vs 9, 4–52 pg/ml, $P = 0.03$) and a trend for increased IL-8 (1258, 35–7500 pg/ml vs 531, 44–3000 pg/ml, $P = 0.06$).

To investigate the potential confounding effect of steroid therapy, we excluded children treated with ICS from the analysis. The main results of our study, with regard to eosinophils and ECP, were confirmed. Indeed, asthmatic children not treated with ICS had increased eosinophilia and ECP levels when compared to control children (0, 0–29% vs 0, 0–9%; $P = 0.003$ and 15, 2–200 μ g/l vs 8, 2–200 μ g/l; $P = 0.01$).

Correlations

In all children, the levels of ECP were positively correlated with eosinophil numbers ($P < 0.0001$ and $r = 0.47$) and

Table 3 Cellular counts and proteins in BAL in atopic and nonatopic asthmatic children

	Atopic asthmatic children (54)	Nonatopic asthmatic children (37)
Total cells ($\times 10^6$ /ml)	0.158 (0.017–14.700)	0.190 (0.040–0.400)
Eosinophils ($\times 10^6$ /ml)	0 (0–1.176)	0 (0–0.055)
Eosinophils (%)	0 (0–29)	0 (0–24)
ECP (μ g/l)	16.8 (2.0–200.0)	9.6 (2.0–200.0)
Eotaxin (pg/ml)*	18.0 (3.2–45.0)†	8.6 (1.8–16.6)
Neutrophils ($\times 10^6$ /ml)	0.013 (0–7.938)	0.020 (0–0.304)
Neutrophils (%)	9 (0–92)	14 (0–76)
IL-8 (pg/ml)*	359 (35–7500)	304 (44–2534)
TNF α (pg/ml)*	9.3 (4.0–66.6)	8.2 (4.0–58.4)

All values are expressed as median (minimum – maximum).

*IL-8 was measured in 44 atopic asthmatic and 30 nonatopic asthmatic children; TNF α in 36 atopic asthmatic and 25 nonatopic asthmatic children; eotaxin in 16 atopic asthmatic and 9 nonatopic asthmatic children.

† $P = 0.005$ vs nonatopic asthmatic children.

percentages ($P < 0.0001$ and $r = 0.44$). These correlations remained significant when only asthmatic children were considered ($P < 0.0001$ and $r = 0.49$; $P = 0.0001$ and $r = 0.47$). Moreover, in all children, BAL eosinophils and ECP levels showed a positive correlation with the number of circulating eosinophils ($P = 0.006$ and $r = 0.33$; $P = 0.01$ and $r = 0.19$). The correlation between BAL eosinophils and circulating eosinophils remained significant when only asthmatic children were considered ($P = 0.02$; $r = 0.31$).

In a subset of patients ($n = 59$), eosinophil counts in bronchial biopsies were available from previously published articles (20, 21, 24). In all these children, tissue eosinophils were correlated with circulating eosinophils ($P < 0.0001$ and $r = 0.54$) and with BAL ECP levels ($P = 0.002$ and $r = 0.40$); however, the correlation with ECP levels was not maintained when considering asthmatic children only.

In all children, the levels of IL-8 and TNF α were positively correlated with neutrophil numbers ($P < 0.0001$ and $r = 0.55$; $P = 0.0001$ and $r = 0.35$) and percentages ($P < 0.0001$ and $r = 0.54$; $P = 0.0009$ and $r = 0.29$). The correlation between neutrophil numbers and IL-8 or TNF α levels remained significant when only asthmatic children were considered ($P < 0.0001$ and $r = 0.52$; $P = 0.03$ and $r = 0.27$).

Discussion

This study examined the markers of eosinophilia and neutrophilia in children with asthma compared to the appropriate control groups. Eosinophils and ECP, but not neutrophils or related mediators, were increased in asthmatic children when compared to controls without atopy, but not when compared to atopic controls. Of interest, the number of eosinophils and the levels of ECP were increased in asthmatic children irrespective of atopic status.

At present, there is a need to better define the airway inflammatory changes in asthmatic and atopic children. Several studies were aimed at evaluating airway inflammation in asthmatic children indirectly by induced sputum and FeNO measurements, or directly by BAL and bronchial biopsies during fiberoptic bronchoscopy. These reports showed the evidence of an increased eosinophilic inflammation not only in induced sputum, but also in BAL fluid and bronchial biopsies (6, 7, 20, 21, 25, 31, 32). Other reports suggested that activation of a neutrophilic response may contribute to the pathophysiology of asthma, at least in a subset of patients (9–12, 33).

Our study confirms and extends those previous observations regarding eosinophilia. Indeed, we found increased eosinophil numbers and percentages, as well as ECP levels, in asthmatic children when compared to nonatopic nonasthmatic controls. As for atopic children without asthma, bronchoscopy studies which evaluated airway inflammation in these subjects are scant. We previously demonstrated that eosinophil numbers in bronchial biopsies of atopic nonasthmatic children are similar to those detected in asthmatic children (20, 21). By contrast, in BAL, Stevenson et al. reported an increased percentage of eosinophils in asthmatics when com-

pared to atopic nonasthmatic children (5). In the present study, asthmatics and atopic nonasthmatics had similar numbers of eosinophils and similar ECP levels, and although the number of eosinophils in atopic nonasthmatics did not reach the level of statistical significance compared to controls, ECP values were definitely increased in atopic children without symptoms of asthma. Of interest, in a subset of patients representative of the three groups in whom bronchial biopsies were available, the number of tissue eosinophils correlated with ECP levels, but not with the eosinophil percentages or numbers in BAL. We should underline that, while the number of eosinophils detected in BAL was extremely low in all groups (median value 0), ECP levels had a much broader range. A plausible explanation for this is that ECP, which may be produced by airway eosinophils, is a protein of very low molecular weight, 16–22 kDa (34) that can easily pass through bronchial epithelium. Therefore, it may be more easily detectable in BAL fluid than eosinophils themselves and could be particularly informative in children with low eosinophil percentages.

When we compared eosinophilic markers in atopic and nonatopic children with asthma, no differences were observed between the two groups with regard to eosinophil numbers, percentages or ECP levels. This observation could be somewhat unexpected, because atopy is a well-known factor for symptoms persistence in wheezing children. Indeed, epidemiological studies have shown that, while a high proportion of nonatopic wheezers resolve their symptoms by adolescence, atopic children have a considerably higher risk for wheezing persistence in adult life (15–19). Of interest, the findings of the present study are consistent with those of our previous one in bronchial biopsies showing that a prominent eosinophilia is present in airway tissue of nonatopic asthmatic children (24). It is important to note that all asthmatic children included in our study had a persistent pattern of symptoms that was not purely viral wheezing, indicating that in the presence of symptoms suggestive of asthma, the inflammation is that typical of the disease even in nonatopic children. The only difference observed in our study between atopic and nonatopic asthmatic children was a significant reduction in eotaxin in nonatopic children. We should highlight, however, that eotaxin measurements were not performed in all children, and the reduced sample size does not allow us to draw definitive conclusions on this point.

When we examined markers of activation of the neutrophilic response, we did not find any difference among asthmatics, atopic and nonatopic controls. Indeed, although there was evidence of neutrophilia in all groups, neither the percentage of BAL neutrophils nor IL-8 or TNF α were increased in asthmatic children. Of note, the previous studies that proposed a role for neutrophils in the pathogenesis of asthma found neutrophilic inflammation to be present in about 1/3 of asthmatic children (9, 11). Moreover, the two previous studies that assessed induced sputum parameters and reported increased neutrophilia were performed in children with acute asthma (35, 36), and exacerbation of symptoms is often driven by infections, which may also increase neutrophilia. In the present study, when inflammatory parameters were compared in children with positive and

negative BAL cultures, increased neutrophilia was indeed present in infected children. We should highlight that children with positive BAL cultures were equally distributed among asthmatics, atopic nonasthmatics and controls, and this can be explained by the fact that recurrent pneumonia was a frequent clinical indication for FB in all groups. Nevertheless, even when children with positive BAL cultures were excluded from the analysis, the main results of our study were confirmed, because asthmatics and atopic nonasthmatics had increased ECP and, among asthmatics, there were no differences between atopic and nonatopic children.

Given the nature of our study, the heterogeneity of our population is a crucial issue. Indeed, children included in our report had a wide age range, and age is an important factor to be accounted for when differentiating asthma from other forms of wheezing and when considering the atopic status. We then performed an age-stratified analysis considering separately infants/toddlers, preschool and school-aged children. We demonstrated that eosinophilia and ECP levels were increased in both school-aged and preschool asthmatic children when compared to controls of the same age and, again, in neither age-frame were there differences between atopic and nonatopic asthmatics. By contrast, there was no evidence of eosinophilia in younger children, in agreement with a study on biopsies that did not find eosinophilia in infants with clinical features of asthma (37). Of interest, there were no differences in neutrophilic inflammation in our study at any time-point, not even among very young children, and this may appear to contrast with some investigations that reported increased neutrophilia in infant/toddler wheezing (9–11). However, we have to acknowledge that our study was not specifically designed to investigate infants, and indeed, there were very

few subjects in this subgroup; therefore, we can not rule out a role for neutrophils in early wheezing.

An important weakness of the study is that a proportion of asthmatic children was in therapy with inhaled beta-2 agonists and inhaled corticosteroids and for ethical reasons, we could not withdraw the current antiasthmatic therapy. However, when we excluded children treated with ICS from the analysis, the main findings of our study were confirmed.

Last but not least, the major limitation in studies of this kind is that all subjects, including the controls, had concomitant diseases, and this may account for lack of increased total cell counts in asthmatics. However, this is an unavoidable constraint, because children had to have a clinical indication to undergo the FB that otherwise would not be permitted for ethical reasons. Although the different indications were equally distributed in the three groups, we can not completely rule out an effect of the concomitant conditions.

In conclusion, this study suggests that markers of eosinophilic inflammation are increased in asthmatic children, but also in atopic children without asthma when compared to nonatopic controls. Somewhat surprisingly, in asthmatic children, activation of the eosinophilic response is not solely because of the presence of atopy. Conversely, neutrophilic activation is not a prominent feature of the inflammatory response in asthmatic or atopic subjects, at least in those older than 3 years.

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