

Increased eosinophil cationic protein levels in bronchoalveolar lavage from wheezy infants

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Although studies examining the serum suggest a role for eosinophils in wheezing episodes in infants and toddlers, the presence of a chronic eosinophilic inflammation within their airways remains to be demonstrated. In this study we investigated whether eosinophil cationic protein (ECP) levels are increased in BAL fluid (BALF) from infants and toddlers with recurrent wheezing episodes, during an asymptomatic period. The levels of ECP in BALF were quantitated by radioimmunoassay in 61 children (36 with severe recurrent episodes of wheezing and 25 who were non-wheezy), aged 6–36 months, in whom flexible bronchoscopy was clinically indicated. BALF eosinophil counts were $\leq 1\%$ in all patients and did not differ in wheezers, compared to non-wheezers. In contrast, ECP levels in BALF were $\geq 2.2 \mu\text{g/l}$ in 18 of 36 (50%) wheezy infants but in only three of 25 (12%) control infants ($p < 0.01$). Neutrophil counts were significantly higher in the wheezer group than in the non-wheezer group (8.1×10^3 cells/ml vs. 3.0×10^3 cells/ml). ECP levels in the BALF were not correlated with the absolute number of eosinophils ($r = 0.03$; $p = 0.8$) but were correlated with the absolute number of neutrophils ($r = 0.54$; $p = 0.001$). There was no association between high ECP levels in BALF and the atopic status of the wheezers. In conclusion, ECP levels are increased in BALF from young children with recurrent wheezing episodes, even during relatively quiescent periods, suggesting a chronic increased cell activation in the lower airways.

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Recurrent wheezing episodes in infants are a major cause of morbidity and hospital admissions in this age group. However, controversies on disease definition and management are far from resolved as they probably represent a heterogeneity of diseases with different etiologies and outcomes and a wide range of severity (1). These recurrent wheezing episodes are clinically similar to asthma attacks and thus it seems reasonable to assume that, as in asthma, chronic inflammation is present within the airways of wheezers and should be treated accordingly. This hypothesis is supported by the good response to preventive inhaled glucocorticoids, with symptomatic improvement, reduced requirements for oral steroids or a decrease in hospital admissions for bronchial obstructions (2,3). Our previous findings, demonstrating an increased spontaneous

release of pro-inflammatory eicosanoids and tumor necrosis factor- α by alveolar macrophages from wheezy children (4,5), also strongly suggest a chronic inflammatory status of the airways. However, there is a need for further clarification of this finding as little published information is available on the presence or nature of this inflammation, and the safety of the prolonged use of inhaled steroids in young children has yet to be established.

Accumulated data, derived mainly from studies in bronchoalveolar lavages (BAL) and bronchial biopsies, have implicated the eosinophil and its granule proteins in the inflammation of the asthmatic airways. Eosinophils are present at increased levels in the blood and airways of asthmatic adults, in numbers that correlate with the disease severity (6). Moreover, although data

are conflicting on the clinical value of monitoring eosinophil cationic protein (ECP) levels, it is well-established that the levels of ECP, the most potent tissue-damaging protein of the eosinophil, are also increased in the serum, sputum, and bronchoalveolar lavage fluid (BALF) of asthmatic adults (6–10). The rarer studies available in children point to a similar role for eosinophils in the pathogenesis of asthma in this age group (11–18).

Studies in young children during acute episodes of wheezing have also shown a disease severity-related increase in ECP levels in the serum and nasopharyngeal secretions (19,20), and thus suggest that eosinophils may also contribute to the acute bronchial obstruction in infants. Recent results reported by Koller et al. (21) in a short follow-up study, indicate that serum ECP measurements during acute wheezing episodes may even have a predictive value for identification of a predisposition to asthma in infants. The findings by Lødrup-Carlsen and colleagues (22), the first to document an increase in serum ECP levels correlated to tidal flow volume responsiveness to salbutamol, in infants and toddlers with recurrent episodes of wheezing in the absence of acute obstruction, suggest that the activation of eosinophils may be chronic. However, Stevenson and colleagues (23) have recently questioned a role for eosinophils in viral-associated wheeze, based on the findings of normal eosinophil numbers in non-bronchoscopic BAL.

In our previous investigations on the BAL of young children with recurrent episodes of wheezing, despite the *in vitro* documentation of an increased release of leukotriene B₄ (LTB₄) by alveolar macrophages, we could not demonstrate the expected increase of eosinophils in BAL (4,5,24). It is possible that eosinophils were not identified in BAL as a result of their activation and, thus, loss of their characteristic granules (25). The aim of the present study was to determine whether ECP, as a marker of cell activation, is present in the airways of infants and toddlers with recurrent wheezy episodes.

Subjects and methods

Study design

Between January 1993 and May 1994, 61 children (6–36 months of age) were selected through questionnaire and consultation of patient clinical records, at the time of a clinically indicated flexible bronchoscopy at the Hôpital Necker-Enfants Malades. The protocol for the study was approved by the hospital's Ethical Committee.

ECP levels in BAL were compared in two groups of children: those with recurrent wheezing and non-wheezy controls.

Population

Thirty-six wheezers (30 boys and six girls; mean age 9.7 ± 0.6 months) were enrolled in the study. They had experienced at least three moderate-to-severe episodes of wheezing. Severity and/or frequency of symptoms (requiring oral corticosteroids more than once a month) and failure of therapy with inhaled steroids led to investigation of airways abnormalities using flexible bronchoscopy. Bronchoscopy was performed during a symptom-free period and inhaled corticosteroids were stopped at least 2 weeks prior to the bronchoscopy.

As it was not ethical to subject normal children to bronchoscopy, the control group were children of the same age range as the children with recurrent wheeze, but without a past or present history of wheezing, who underwent flexible bronchoscopy for investigation of other pulmonary diseases during the same period. The control group (19 boys and six girls, mean age 19.9 ± 1.9 months) underwent bronchoscopy for investigation of pulmonary tuberculosis ($n=13$), non-wheezy bronchitis ($n=6$), persistent pulmonary infiltrate ($n=3$), bronchiectasis ($n=2$), or the suspicion of foreign body aspiration ($n=1$). Except for one child who had a history of cow's milk protein intolerance, none had a history of personal or familial atopy. Although the age range was narrow, the controls were significantly older than the wheezers (median 22.1 months vs. 9.4 months, $p < 0.01$). This difference was taken into account during the analysis by subdividing the children into two age groups: 6–12 months and 13–36 months.

Children with known cystic fibrosis, congenital or acquired immune deficiencies, congenital malformations, bronchopulmonary dysplasia, or previous ventilation, were excluded from the study. Other exclusion criteria were signs of respiratory distress or a transcutaneous oxygen saturation less than 95% at the time of bronchoscopy, or treatment with glucocorticoids or β -adrenergic agonists during the 2 weeks prior to the study.

An atopic predisposition was investigated among the population of wheezers. A history of familial atopy was considered as positive if there were atopic-related diseases (allergic rhinitis, asthma, or eczema) in one or both parents, or in siblings. Allergy tests were performed and interpreted as previously described (26,27).

Table 1. Clinical data from wheezers

No.	Gender	Age (months)	Familial atopy	Atopic status
1	M	7	+	+
2	M	14	–	+
3	F	6	–	–
4	M	6	–	–
5	M	7	+	–
6	M	23	+	+
7	M	6	–	–
8	M	6	+	–
9	M	11	+	–
10	M	10	–	–
11	F	10	+	–
12	M	16	+	+
13	M	10	+	–
14	M	9	+	–
15	M	13	+	–
16	M	12	–	–
17	F	6	–	–
18	M	9	+	?
19	M	6	–	+
20	M	13	–	?
21	M	11	–	–
22	M	10	+	–
23	M	10	+	+
24	M	9	–	+
25	M	14	–	?
26	M	7	+	+
27	M	6	+	–
28	M	12	–	–
29	M	6	+	+
30	M	8	+	?
31	M	7	–	–
32	F	16	–	–
33	F	6	+	+
34	F	9	–	–
35	M	12	–	?
36	M	10	+	–

F, female; M, male.

Children were classified as atopic if they had at least one of the following parameters: a history of atopic dermatitis diagnosed by a doctor, or increased total serum immunoglobulin E (IgE) levels (28) plus positive skin tests for common allergens. Table 1 summarizes the clinical characteristics of the wheezers in regard to their atopic status. In five patients, allergy tests had been performed in another hospital and, as there is no consensus on the methods of performing and interpreting skin tests in young children (26), these five patients were excluded from the analysis when the association between ECP values and atopy was studied.

Bronchoscopy and BAL

Children received atropine and midazolam as premedication, and upper and lower airway anesthesia was obtained with 2% and 0.5% lidocaine, respectively. The Olympus BF3C30

bronchofiberscope (Olympus Winter IBE GMBH, Hamburg, Germany) was wedged into a sub-segmental bronchus of the right lower lobe, and prewarmed sterile saline, 4 ml/kg of body weight, was instilled in 10-ml aliquots and withdrawn by gentle aspiration. The first aliquot was sent for viral analysis by immunofluorescence, and the remaining recovered fluid was immediately filtered through two layers of sterile surgical gauze, collected into sterile polypropylene vials (Falcon 50 ml; Becton-Dickinson, Rutherford, NJ, USA), and kept on ice until centrifugation.

ECP measurement

The lavage fluid was centrifuged at 470 g for 10 min at 4°C, and BALF was then frozen at –70°C for subsequent measurement of ECP. The ECP level was then determined by radioimmunoassay (kindly provided by Veronique Jamagne,

Kabi Pharmacia, Pharmacia Diagnostics AB, Uppsala, Sweden), according to the manufacturer's instructions (29). Briefly, ECP in the samples competes with ^{125}I -ECP for the binding sites of specific antibodies. Bound and free ECP are then separated by addition of a second immunosorbent antibody, followed by centrifugation and decanting. The radioactivity present in the pellet was counted using a Kontron Analytical gamma counter (model MDA 312, Kontron, Zurich, Switzerland). According to the manufacturer, the cross-reactivity with eosinophil protein X is $<0.06\%$. The lower limit of detection of this method is $2\text{ }\mu\text{g/l}$.

Additional methods

Total cell counts were evaluated in a sample of BAL by using a Malassez hemocytometer (Preciss, Paris, France). Cell viability was determined by using the Trypan Blue dye-exclusion test. Differential cell counts were obtained by modified Wright-Giemsa staining (Diff-Quik, Baxter Dade AG, Duedingen, Switzerland) of cytocentrifuged smears. At least 300 cells were examined by the same observer for each specimen. For mast cell counts, smears were stained with 1% Alcian Blue, after fixation in Carnoy's fluid.

The protein content of BALF was determined using the bicinchoninic (BCA) protein assay kit (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard.

Statistical analysis

Values are presented as medians, with the interquartile range in parentheses. Comparison

between groups was performed by using the Mann-Whitney two-sample *U*-test for unpaired data. Unless stated otherwise, the categorical variables were analyzed by the chi-square test using the Yates' continuity correction, or by Fisher's exact test, where appropriate. Correlations were evaluated using Spearman's rank correlation coefficient test. A *p*-value of less than 0.05 was accepted as significant.

Results

Flexible bronchoscopy and BAL

Bronchoscopy and BAL were well tolerated both by wheezy and control children, and no complications were observed (30). Fluid recovery averaged 65% (range 40–80%) and did not differ between the groups (30). The viability of BAL cells was similar both in wheezy and in control children: 65% (58–80%) vs. 67% (55–77%); and epithelial cells were always non-viable.

The total and differential cells counts of the BAL are presented in Table 2. Eosinophil counts were $\leq 1\%$ in all subjects, and did not differ in wheezers, compared with non-wheezers. The total number of cells recovered in BAL, and the absolute numbers of neutrophils and epithelial cells, were significantly higher in the wheezy infants than in the controls ($p < 0.05$). Mast cell numbers were always below 0.08%.

ECP levels in BAL

As the levels of ECP in BALF were often below the lowest concentration used in the standard curve of the assay ($2\text{ }\mu\text{g/l}$), and allowing for a maximal 10% inter-assay coefficient of variation,

Table 2. Cellular contents of bronchoalveolar lavage (BAL)

	Wheezers	Controls	<i>p</i> -value
Total number of cells			
Cells $\times 10^3/\text{ml}$ of BAL	380 (268–508)	263 (200–384)	0.03
Alveolar macrophages			
Cells $\times 10^3/\text{ml}$ of BAL	313.0 (223.7–404.1)	253.3 (145.4–326.8)	0.09
% of nucleated cells	84.5 (74.5–89.5)	84.9 (79.9–90.1)	0.46
Lymphocytes			
Cells $\times 10^3/\text{ml}$ of BAL	15.8 (12.1–33.0)	19.3 (12.0–34.8)	0.51
% of nucleated cells	4.8 (3.0–7.0)	8.0 (4.3–11.5)	0.02
Neutrophils			
Cells $\times 10^3/\text{ml}$ of BAL	8.1 (2.0–32.9)	3.0 (0.6–6.0)	0.02
% of nucleated cells	2.2 (0.6–9.0)	1.0 (0.3–2.0)	0.06
Eosinophils			
Cells $\times 10^3/\text{ml}$ of BAL	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.73
% of nucleated cells	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.81
Epithelial cells			
Cells $\times 10^3/\text{ml}$ of BAL	19.5 (13.5–26.8)	15.7 (7.3–20.6)	0.04
% of nucleated cells	5.4 (2.8–7.2)	4.9 (2.9–7.7)	0.77

Data are expressed as median values, with ranges from 25th to 75th percentiles in parentheses.

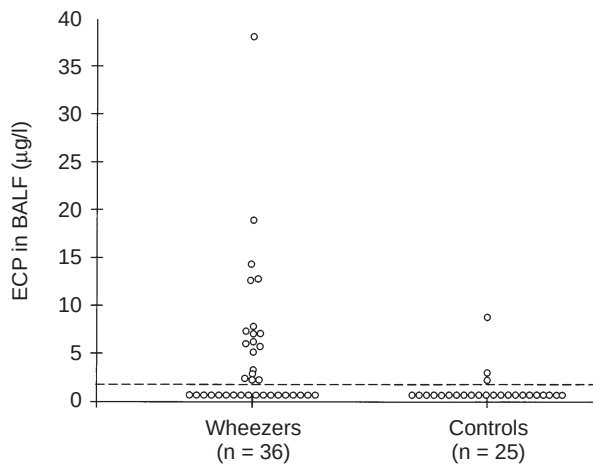


Fig. 1. Eosinophil cationic protein (ECP) levels in bronchoalveolar lavage fluid (BALF) from wheezy and control children. The ECP levels were significantly increased in wheezers, as compared to controls ($p < 0.01$). The dashed line represents the lowest concentration used in the standard curve of the assay.

ECP levels of $< 2.2 \mu\text{g/l}$, or $\geq 2.2 \mu\text{g/l}$, were grouped together.

The ECP levels in BALF were $\geq 2.2 \mu\text{g/l}$ in 18 of 36 (50%) wheezy infants, but in only three of 25 (12%) control infants ($p < 0.01$). This difference remained significant when the results were age-adjusted ($p < 0.025$, Mantel-Haenszel chi-square test). When compared with the controls, the children with wheezing were more likely to have a detectable level of ECP in BALF (odds ratio [OR] Mantel-Haenszel = 4.9) and the risk seemed to increase with age (OR 2.3 and 22.7, respectively, in the younger and older children).

The ECP levels in BALF were not correlated with the absolute number of eosinophils ($r = 0.03$; $p = 0.8$) but were correlated with the absolute number of neutrophils ($r = 0.54$; $p = 0.001$).

Figure 1 illustrates the greater variation of BALF ECP values observed in the wheezers: the values ranged from < 2.2 – $38.0 \mu\text{g/l}$ compared with < 2.2 – $8.6 \mu\text{g/l}$ in the control children. The ECP level was clearly elevated only in one control infant, who had recurrent non-wheezy bronchitis and a history of cow's milk protein intolerance.

The high ECP levels observed in the BALF from wheezers were not associated with atopic

status ($p = 0.13$) (Table 3) nor with a background of familial atopy (Table 4).

We did not correct ECP levels in BALF for the dilutional effect of BAL on the epithelial lining fluid because there is no consensus on a marker for this effect (18,31). Nevertheless, the total protein content of BALF was similar in wheezy and non-wheezy children: $151 \mu\text{g/ml}$ (range 115–180 $\mu\text{g/ml}$) vs. $126 \mu\text{g/ml}$ (range 94–176 $\mu\text{g/ml}$), respectively, $p = \text{NS}$. The individual levels of ECP vs. the total protein content of BALF are plotted in Fig. 2.

Discussion

In the present study we demonstrated that ECP levels are increased in the BALF from young children with severe recurrent episodes of wheezing, as compared with non-wheezy children, despite similarly low eosinophil counts in both populations (18,32). Our findings suggest that eosinophils may be chronically activated in the airways of infants and toddlers with recurrent wheezing episodes. Other indexes attesting to eosinophil activation, such as major basic protein and eosinophil protein X, would have provided more arguments in favor of this conclusion, but were not investigated in our study. Several hypotheses might account for the lack of correlation between ECP levels and the number of eosinophils in BALF. First, the ECP concentration in BALF could reflect the presence of activated eosinophils in the lamina propria (33,34). Second, activated and degranulated eosinophils may not be detected when a counting method dependent on staining is used (34). Third, lysates of purified neutrophils have been shown to contain eosinophil-derived neurotoxin (EDN) and ECP. ECP was detected in neutrophils when a kit from Pharmacia Diagnostics was used (35,36). Although neutrophils contain only 4.5% of the quantity of ECP present in eosinophils, caution should be exercised in interpreting the presence of ECP as a specific marker of eosinophil-associated inflammation in human disease (37). Indeed, a statistically significant increase in neutrophils has been demonstrated in

Table 3. Lack of association in wheezers between atopic status and eosinophil cationic protein (ECP) levels in bronchoalveolar lavage fluid (BALF)

ECP	Atopy status (n=31*)	
	Yes	No
$\geq 2.2 \mu\text{g/l}$ (n=15)	7	8
$< 2.2 \mu\text{g/l}$ (n=16)	3	13
Total	10	21

*In the remaining five of the 36 wheezers the atopy status could not be assessed (in three, ECP levels were $\geq 2.2 \mu\text{g/l}$).

Table 4. Lack of association in wheezers between familial atopy and eosinophil cationic protein (ECP) levels in bronchoalveolar lavage fluid (BALF)

ECP	Familial atopy (n=36)	
	Yes	No
≥2.2 µg/l (n=19)	10	9
<2.2 µg/l (n=17)	9	8
Total	19	17

BALF from infantile wheezers (18,31,38), and the proportion of neutrophils was related to symptom severity (18). It is therefore probable that neutrophils contribute to the pathophysiology of airway disease (36).

Owing to the lack of an agreed method to overcome the variable dilution effect on ECP levels in BALF in ill humans (18,31,39,40), our results should be analyzed with caution. Nevertheless, it appears unlikely that the difference observed in ECP levels in BALF between wheezers and non-wheezers would be entirely attributable to a dilutional effect of BAL. In our study, the amount of fluid used in the BAL was adjusted to the size of the child. This approach has been validated by Ratjen & Bruch (41) as yielding reasonably consistent fractions of the epithelial lining fluid in children. Furthermore, the percentage recovery of BAL was similar in wheezers and non-wheezers, and there were no differences in the protein content of BALF between the two groups. The absence of correlation between ECP levels and protein levels is in agreement with the results of Ennis et al. (16).

The demonstration of variation within the ECP values in BALF from wheezers may reflect the

heterogeneity of etiologies and outcomes of wheezing episodes in young children (1,17). It is possible that the ECP levels in BALF are increased in those who will continue to wheeze, a hypothesis that will require longitudinal studies. Nevertheless, the range of ECP values in BALF from wheezers is of the same order of magnitude of the levels documented in older asthmatics (6,9–11,17).

In our study, a possible association between ECP levels in BALF and disease severity could not be evaluated owing to the narrow range of disease severity within the study population. All subjects had relatively severe forms of disease that justified invasive diagnostic methods in the search for airways abnormalities, and all patients were studied during asymptomatic periods. This also highlights that our results cannot necessarily be extrapolated to the entire population of wheezy infants. However, the wide range of ECP values in BALF from patients of identical clinical severity suggests that its measurement is of limited value for the clinical evaluation of the individual patient.

Atopy has been suggested as a factor that enhances eosinophil activity in asthmatic children (12,14,17). Although atopy is difficult to document by classical criteria in infants and toddlers, we investigated whether an association was present between increased ECP levels in BALF and a positive familial background of atopy, or between increased ECP levels in BALF and the presence of atopic dermatitis or increased serum IgE levels plus positive skin tests for common allergens. We were unable to demonstrate a significant association between atopy and high levels of ECP in BALF. However, the children in our study were not symptomatic when BAL was performed, and we therefore cannot exclude that a difference may be present in the BALF ECP levels between atopics and non-atopics during an acute episode of wheezing. Zimmerman and colleagues (12) could only demonstrate an increase in serum ECP levels in atopic vs. non-atopic young asthmatic children in the presence of asthma symptoms.

Stevenson and colleagues (23) have suggested (based on the presence of normal eosinophil and

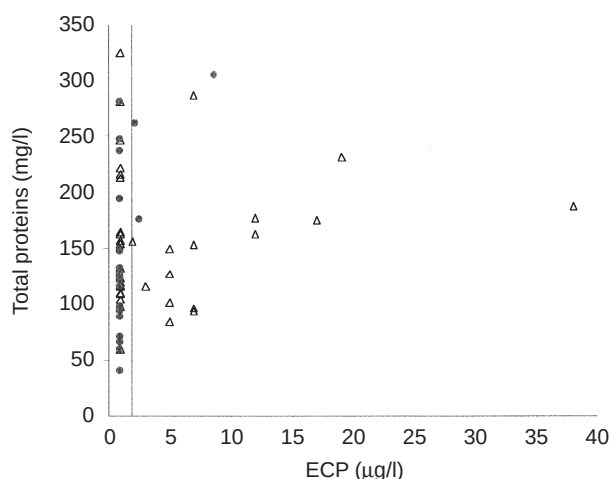


Fig. 2. Individual eosinophil cationic protein (ECP) level vs. total protein level in bronchoalveolar lavage fluid (BALF) from wheezy and control children. The dashed line represents the lowest concentration of ECP used in the standard curve of the assay. Δ , wheezers ($r=0.06$, $p=0.7$); $\bullet$, controls ($r=0.38$, $p=0.06$).

mast cell numbers in non-bronchoscopic BAL procedures) that ongoing airways inflammation does not occur in children with viral-associated wheeze. In a recent study (16), the same group found significantly higher levels of ECP in atopic asthmatic children than in children with viral-associated wheeze. In this latter group, only four of 21 children had high values of ECP in BALF. The difference between their results and ours might be because of the greater heterogeneity in age (3.6–13 years) and severity of their population.

In our study, the higher concentration of ECP in BALF from wheezers may indirectly reflect airway inflammation. Further studies are required to investigate whether increased levels of pro-inflammatory mediators in BALF reflect an inflammatory status within the lung. Nevertheless, the findings of this study are in accordance with those of our previous work, in which we demonstrated an increased spontaneous release of pro-inflammatory mediators by alveolar macrophages from wheezy children (4,5). The increases in total cellularity and absolute numbers of neutrophils and epithelial cells in BAL from wheezers also favor this hypothesis. The higher epithelial cell numbers in BAL may result from increased epithelial shedding into the airways of wheezy children, as a result of exposure of the epithelium to ECP. However Marguet and colleagues have recently shown that epithelial damage might occur independently of eosinophilic inflammation (18). Furthermore, we cannot reject the fact that this increase could result from an increased percentage of bronchial fluid vs. alveolar fluid in the BAL recovered from wheezers (42).

In conclusion, ECP levels in BALF from young children with recurrent wheezing episodes are significantly higher than those measured in controls, suggesting that eosinophils, and possibly neutrophils, may contribute to the pathogenesis of wheezing in infancy. Our findings confirm the presence of an ongoing inflammatory process in the airways, even during quiescent periods, in a sub-group of wheezy infants with frequent, recurrent symptoms.

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