

Bronchoalveolar lavage eosinophil cationic protein and interleukin-8 levels in acute asthma and acute bronchiolitis

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Summary

Objective In this study, we measured the levels of eosinophil cationic protein (ECP) and interleukin (IL)-8 in bronchoalveolar lavage (BAL) fluid from patients with acute asthma and acute bronchiolitis, to determine any similarities or dissimilarities in the profiles of these biochemical markers in the two diseases.

Methods BAL fluids were obtained from children with acute asthma ($n = 16$), infants with acute bronchiolitis caused by respiratory syncytial virus ($n = 18$), and control subjects ($n = 14$). Children with asthma were selected to be free of viral infection. BAL cell counts and differentials were determined, and ECP and IL-8 levels were measured by radioimmunoassay and ELISA, respectively.

Results ECP levels in BAL fluids were significantly higher in the asthma group than in the bronchiolitis ($P < 0.01$) or control ($P < 0.0001$) groups. However, IL-8 levels were significantly higher in the bronchiolitis group than in the asthma ($P < 0.01$) or control ($P < 0.001$) groups. IL-8 levels in the asthma group and ECP levels in the bronchiolitis group were similar to those of the control group.

Conclusion This difference in profiles of ECP and IL-8 in acute asthma and acute bronchiolitis, together with a different inflammatory cell pattern, suggests that the nature of the inflammatory process within the lower respiratory tract may be distinctive in these two diseases.

Keywords acute asthma, acute bronchiolitis, bronchoalveolar lavage, eosinophil cationic protein, interleukin-8

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Introduction

Infection by respiratory syncytial virus (RSV) in infancy frequently causes bronchiolitis [1], which has been associated with an increased risk for the development of recurrent wheezing [2] or asthma [3] later in childhood. In line with the clinical analogy and the epidemiological relationship between the two diseases, several studies have suggested that acute bronchiolitis has certain features in common with asthma, such as specific IgE production [4], chemokine generation [5] and elevated levels of adhesion molecules [6]. However, the pathogenetic basis for the relationship between these two diseases has not been completely elucidated.

Bronchoalveolar lavage (BAL) is a valuable research tool [7] and there is a growing interest in its use in children [8]. We recently demonstrated that acute asthma and acute bronchiolitis BAL fluids were characterized by elevated percentages of eosinophils and neutrophils, respectively [9]. This finding suggests that acute asthma and acute bronchiolitis are associated with different types of cellular inflammation. To

our knowledge, however, BAL fluids have not been evaluated for distinctive biochemical markers of airway inflammation in these two diseases.

Eosinophil cationic protein (ECP), one of the secretory proteins of eosinophils, has been suggested as a marker of eosinophil activation in asthma [10], and levels of ECP have been found to correlate with disease activity [11]. The pathogenic mechanisms of neutrophil inflammation include an influx in response to the chemokine interleukin (IL)-8, with subsequent release of several potent tissue-damaging pro-inflammatory enzymes, such as myeloperoxidase [12]. Thus, ECP and IL-8 may be considered to, respectively, reflect eosinophil- and neutrophil-mediated airway inflammation. However, circumstantial evidence suggests that these relationships are not straightforward. For example, a recent report found that ECP localizes to neutrophils [13], and neutrophils have been observed to take up and store the ECP released by eosinophils [10]. In fact, elevated levels of ECP have been found in the absence of eosinophils in other neutrophil-mediated airway diseases such as bronchiectasis [14] and chronic obstructive airway disease [15]. On the other hand, IL-8 may potentiate eosinophil recruitment and activation, and evidence suggests that IL-8 can function as a chemotactic factor for cytokine-primed eosinophils [16]. Dose-dependent migration of eosinophils in response to IL-8

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has also been demonstrated [17]. Correlations between IL-8 levels and eosinophil numbers have been demonstrated in the induced sputum of patients with moderate asthma [18].

In this study, we measured the levels of ECP and IL-8 in BAL fluid from patients with acute bronchiolitis and from those with acute asthma where viral infection has been excluded, to determine whether there is a distinguishing feature in the profiles of these biochemical markers in the two diseases.

Methods

Study population

The study subjects were 16 patients with acute asthma, 18 patients with acute RSV bronchiolitis, and 14 control subjects, all of whom underwent flexible bronchoscopy (FB) with BAL from July 1998 to July 2001 either as part of the approved study protocol or for clinical indications. Nasopharyngeal aspirates (NPAs) were screened for viral infections, by indirect immunofluorescence, for six respiratory viruses (RSV, adenovirus, influenza viruses A and B, and parainfluenza 1 and 3) in all three study groups at the time of entry. Complete history taking, a physical examination, and routine blood tests were performed for all subjects at the time of entry.

The asthma group consisted of 10 boys and six girls (median age, 3.3 years; range, 1.9–4.5 years) who were hospitalized because of an acute asthma exacerbation. All children in this group had been diagnosed as having asthma on the basis of recurrent wheezing and dyspnoea attacks with proven β_2 -agonist effects [19]. All of them were atopic, as defined by positive skin prick test response to at least one of the 14 common airborne allergens. All children had used bronchodilators on demand during symptomatic periods. Eight of the 16 had also received prophylactic therapy (i.e., inhaled glucocorticoids or cromoglycate). All 16 had no signs of viral or bacterial infections at the time of admission, as evaluated by clinical examination, chest X-ray, serum C-reactive protein measurement, serologic test for *Mycoplasma*, and viral antigen tests in NPAs. Initially, 22 patients with asthma were enrolled, however, six were positive by the viral antigen test and were excluded. None of the subjects had a positive viral culture in BAL fluid. During hospitalization, all subjects were medicated with regular nebulized albuterol (0.5 mL of a 0.5% solution diluted with 2 mL of saline q 4–6 h) and systemic corticosteroids (2 mg/kg of methylprednisolone or prednisolone).

The RSV bronchiolitis group consisted of 10 boys and eight girls (median age 1.3 years; range 0.5–2.3 years). All of these subjects required hospitalization for the treatment of their first episode of bronchiolitis. Bronchiolitis was defined as an acute respiratory illness with tachypnoea, dyspnoea, wheezing and fine crepitation on auscultation [20]. Infants with atopic dermatitis or a family background of atopy were excluded. RSV infection was ascertained by the identification of viral antigens in NPAs [21] and/or the recovery of the virus in cell culture. For four infants, BAL cultures were positive for RSV, although the results of the viral antigen tests were negative [22]. All subjects in this group received conventional

therapy with ribavirin and no corticosteroids during their hospitalizations.

Fourteen children who were admitted with indications for FB (i.e., lung mass, stridor or localized emphysema) served as control subjects (median age, 3.7 years; range, 1.7–4.2 years). None of them had a history of asthma or allergic diseases. They had no identifiable airway infections in the 4 weeks prior to the study. In all subjects, both NPA antigen test and BAL fluid culture were negative for viruses.

Parents of the patients and of the control subjects gave informed consent, and the study was approved by the Hospital Ethics Committee.

Flexible bronchoscopy with bronchoalveolar lavage

FB with BAL was performed on the 4th day after admission, with a 3.6 mm pediatric flexible bronchoscope (Olympus BF-3C30; Olympus, New Hyde Park, NY, USA). Nebulized albuterol was prescribed uniformly before the procedure. Premedication consisted of intramuscular atropine sulfate (0.01–0.02 mg/kg, to a maximum of 1 mg) and intravenous midazolam (0.1–0.2 mg/kg). Subjects were also given nebulized albuterol and lidocaine (titrated by weight) to anaesthetize the upper airway. During bronchoscopy, oxygen and epinephrine were readily available, and subjects had an intravenous line to provide venous access. Heart rate and transcutaneous SaO_2 were monitored throughout the procedure and continued for 1 h. After examination of the upper and lower airways, the tip of the bronchoscope was wedged into a segmental or subsegmental bronchus of the right middle lobe. Three 1 mL/kg aliquots of sterile, non-bacteriostatic saline, at room temperature, were instilled through the instrumentation channel. Each aliquot was immediately aspirated into a sterile specimen container using a wall suction pressure of 100–150 mmHg. BAL fluid aspirated after each instillation was pooled and immediately placed on ice.

Processing and analysis of bronchoalveolar lavage fluid

The total amount of recovered fluid was measured and recovery was calculated as a percentage of the volume instilled. Pooled BAL fluid was split into two aliquots. One aliquot was submitted for viral and bacterial cultures to the Hospital Microbiology Department. Standard culture techniques were used to isolate six respiratory viruses (RSV, adenovirus, influenza A and B, and parainfluenza 1 and 3) and common respiratory bacteria in the BAL fluids of all three groups. The remainder of the pooled BAL fluid was taken to the laboratory for analysis of the cellular and fluid fractions. In order to avoid spuriously high levels of ECP or IL-8 because of the release as a consequence of the exposure of cells to activating agents, the fluid was immediately processed by centrifuging at 400 *g* for 10 min at 4 °C to separate fluid from cells. Total cell count was determined, using a haemocytometer (Weber, Teddington, UK). Viability was assessed by the trypan blue exclusion test. The cellular composition of BAL fluid was determined using cytopsin (Shandon, Pittsburgh, PA, USA) slide preparations with a May–Grunwald–Giemsa stain and by calculating percentages on 400 cell counts. The cell-free fluid was frozen at –70 °C for mediator assay.

Concentration of the bronchoalveolar lavage fluid

Because of the large dilution effect caused by the instilled fluid and the low levels of ECP expected, BAL fluid was concentrated using Centricon-10 concentrators (Amicon Co., Beverly, MA, USA) with a molecular weight cutoff of 10 kDa, according to the manufacturer's recommendations. Briefly, a 0.5 mL aliquot of BAL fluid per patient was centrifuged at 4900 *g* in a Sorvall ultracentrifuge (Du Pont Co., Wilmington, DE, USA). Several centrifuge cycles were performed. A 100 µL volume was retained, which corresponded to a fivefold concentration.

Mediator assays

ECP was measured in fivefold concentrated BAL fluids by radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) at a detection limit of 2 µg/L. Concentrations below the detection limit were assumed to be 2 µg/L for the purpose of statistical analysis. IL-8 was measured in unconcentrated BAL fluids using an ELISA kit (CLB) with a detection limit of 2 pg/mL. All assays were run in duplicate and mean values were used for statistical analysis.

Statistical analysis

ECP levels are expressed as micrograms per liter in concentrated BAL fluid and IL-8 levels as picograms per milliliter in unconcentrated BAL fluid; both ECP and IL-8 levels are presented as medians with the interquartile range [25th–75th]. Cellularity data are expressed as numbers per milliliter in unconcentrated BAL fluid (total cell count) or as percentages of the total cell count (cell differential), and are presented as medians with the interquartile range. As most of the data were not normally distributed, non-parametric analysis was used. Screening of the data for differences in BAL mediators and cell profiles in the study groups was performed using the Kruskal–Wallis test. When significant differences were identified, individual groups were compared using the Mann–Whitney *U*-test. Correlations between values were assessed by calculating Spearman's correlation coefficients. A *P*-value of <0.05 was considered statistically significant.

Results

Subject characteristics

The characteristics of the subjects studied are shown in Table 1. Comparisons of the three groups showed that children with RSV bronchiolitis were significantly younger than the

children in the other two groups, and that those with asthma had significantly higher serum IgE and blood eosinophil counts.

Procedure-associated complications

FB with BAL was relatively well tolerated by all subjects. Minor procedure-associated complications occurred on eight occasions, comprising transient hypoxia (*n* = 4), mild bronchospasm (*n* = 2), transient bradycardia (*n* = 1) and minor epistaxis (*n* = 1). These complications, however, did not preclude the completion of the procedure. Three children had a transitory hoarse cough and four others had a low grade fever of up to 38 °C, which occurred within 12 h of the procedure. These adverse events resolved spontaneously within 24 h.

Bronchoalveolar lavage cell profiles

The recovery of BAL fluid, cell viability, the total cell numbers per milliliter, and the percentages of each cell type are shown in Table 2. No differences were found in the recovery of BAL fluid or in cell viability between the groups. Total cell numbers were significantly higher in the bronchiolitis group than in the asthma or the control groups. There were significant differences in the percentages of macrophages, neutrophils, and eosinophils among the three groups. The neutrophil percentages in the bronchiolitis group were significantly higher than in the asthma group or the control group. On the other hand, the eosinophil percentages in the asthma group were significantly higher than in the other two groups. The percentage of macrophages was lower in the asthma and bronchiolitis groups compared with the control group, which occurred in conjunction with a marked increase in the percentage of eosinophils or neutrophils. All other cell types showed no significant differences among the groups.

Bronchoalveolar lavage mediator levels

Eosinophil cationic protein levels The levels of ECP in the concentrated BAL fluid of the study groups are shown in Fig. 1. The ECP level was significantly higher in the asthma group (median with interquartile range [25th–75th], 20.5 [10.5–48.7] µg/L) than in the bronchiolitis (7.1 [4.1–9.1] µg/L, *P* < 0.01) and control groups (4.8 [2.0–7.5] µg/L, *P* < 0.0001). ECP levels were undetectable in four controls. No significant differences in ECP levels were noted between the bronchiolitis and control groups.

IL-8 levels The levels of IL-8 in the unconcentrated BAL fluid of the study groups are shown in Fig. 2. The IL-8 level

Table 1. Baseline characteristics of the subjects

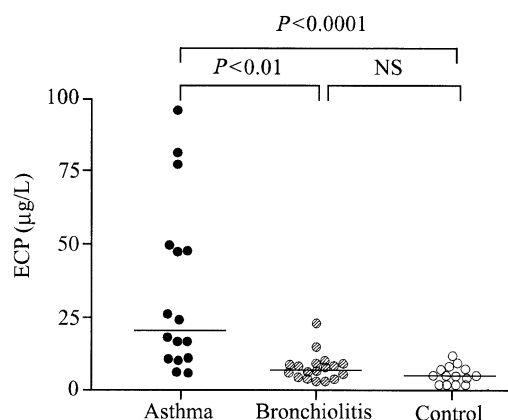
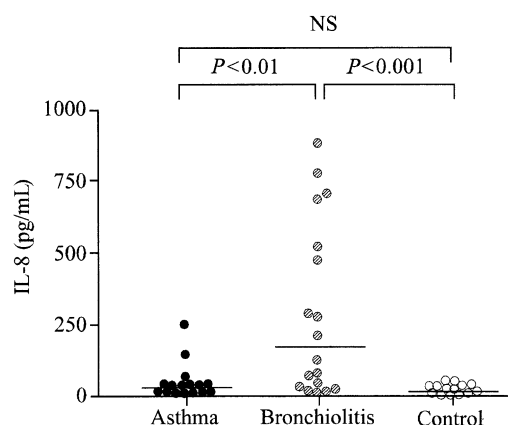
	Asthma group (<i>n</i> = 16)	Bronchiolitis group (<i>n</i> = 18)	Control group (<i>n</i> = 14)
Age (years)*	3.3† (1.9–4.5)	1.3‡ (0.5–2.3)	3.7 (1.7–4.2)
Sex (M:F)	10:6	10:8	9:5
Serum IgE (IU/mL)*	246†‡ (120–1,812)	33 (5–106)	58 (21–133)
Blood eosinophils (/mm ³)*	520†‡ (150–1,012)	114 (28–388)	146 (30–231)
Blood neutrophils (× 10 ³ /mm ³)*	3.2 (1.3–4.2)	3.6 (1.8–7.1)	2.8 (1.2–3.9)

*Data are expressed as median (range). †*P* < 0.01 compared with bronchiolitis; ‡*P* < 0.01 compared with control.

Table 2. Bronchoalveolar cell profiles of the three groups of subjects*

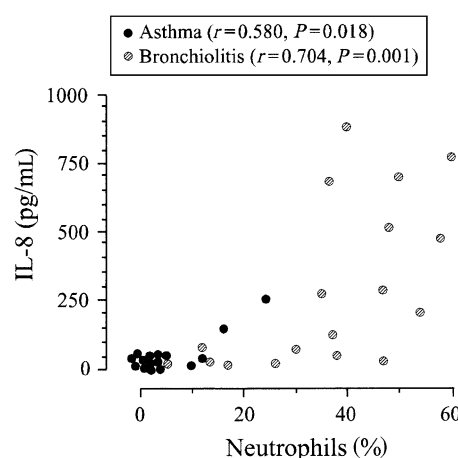
	Asthma group (<i>n</i> = 16)	Bronchiolitis group (<i>n</i> = 18)	Control group (<i>n</i> = 14)
Recovery (%)	42.0 [35.0–45.0]	39.5 [34.5–48.5]	45.5 [38.8–52.0]
Viability (%)	85.0 [79.0–89.8]	83.0 [76.0–88.3]	86.5 [80.5–90.3]
Total cells ($10^4/\text{mL}$)	25.0 [14.3–28.0]	33.0 ^{†‡} [19.3–63.5]	16.0 [9.1–20.5]
Macrophages (%)	78.0 [‡] [71.3–83.8]	55.0 ^{†§} [45.8–63.5]	86.5 [82.0–89.0]
Lymphocytes (%)	9.3 [5.0–15.0]	5.0 [3.0–8.5]	8.0 [5.8–10.5]
Neutrophils (%)	3.3 [2.0–8.8]	37.5 ^{‡§} [23.8–48.5]	2.3 [1.0–4.3]
Eosinophils (%)	2.1 ^{‡¶} [2.0–4.3]	0 [0–1.3]	0 [0–0]
Epithelial cells (%)	3.0 [2.0–3.9]	2.0 [1.5–3.0]	2.0 [1.0–3.1]

*Data are expressed as median and interquartile range [25th–75th]. [†] $P < 0.01$ compared with asthma; [‡] $P < 0.001$ compared with control; [§] $P < 0.001$ compared with asthma; [¶] $P < 0.001$ compared with bronchiolitis.

**Fig. 1.** Eosinophil cationic protein (ECP) levels in concentrated bronchoalveolar lavage fluid from the three groups. Median values are represented as horizontal bars.**Fig. 2.** Interleukin (IL)-8 levels in bronchoalveolar lavage fluid from the three groups. Median values are represented as horizontal bars.

was significantly higher in the bronchiolitis group (median with interquartile range, 166.0 [29.2–558.8] pg/mL) compared with the asthma group (21.6 [10.8–45.9] pg/mL, $P < 0.01$) and the control group (14.2 [8.5–21.8] pg/mL, $P < 0.001$). No significant difference in IL-8 levels was observed between the asthma and control groups.

Correlations We investigated the correlations between the ECP levels and the eosinophil percentages, and between the

**Fig. 3.** Correlation between interleukin (IL)-8 levels and neutrophil percentages in the bronchoalveolar lavage fluids of the asthma group and the bronchiolitis group.

IL-8 levels and neutrophil percentages in the BAL fluids. IL-8 levels correlated significantly with neutrophil percentages in both the asthma group ($r = 0.580$, $P = 0.018$) and the bronchiolitis group ($r = 0.704$, $P = 0.001$) (Fig. 3). However, ECP levels were not correlated with the eosinophil percentages in either the asthma group ($r = 0.396$, $P = 0.128$) or the bronchiolitis group ($r = 0.179$, $P = 0.753$) (data not shown). We also investigated cross correlations between the ECP levels and neutrophil percentages, and between the IL-8 levels and eosinophil percentages. None of the cross correlations was statistically significant in either the asthma group ($r = 0.449$, $P = 0.081$; $r = 0.333$, $P = 0.207$) or the bronchiolitis group ($r = 0.385$, $P = 0.115$; $r = 0.284$, $P = 0.253$) (data not shown).

Discussion

This study presents the results of the first comparison of ECP and IL-8 concentrations in BAL fluid from children with acute asthma and acute bronchiolitis. High ECP levels with an increase in eosinophil count were observed in children with acute asthma. On the other hand, children with acute bronchiolitis showed markedly elevated levels of IL-8 and these correlated significantly with neutrophil numbers. ECP

levels in children with acute bronchiolitis and IL-8 levels in children with acute asthma, however, were no different from those of the control children.

In the present study, samples were obtained using a standardized BAL technique. The region of lung sampled by FB with BAL depends on the size of the child, for anatomic and technical reasons [23]. The effects of sampling bronchi in different orders would be reflected in the first aliquot [24]. Therefore, most authors discard this for cellular studies. However, we pooled all specimens, including the first BAL aliquot, since the concentrations of acellular components would progressively decrease in sequential aspirates [25]. This procedural difference explains the higher total cell counts and percentages of epithelial cells found in the present study as compared with our previous study [9]. When the cellular compositions of BAL fluids were compared between the study groups, neutrophils were found to be the predominant cells in acute bronchiolitis, whereas increased eosinophil numbers was the most consistent finding in acute asthma. These findings confirm the results of our previous study [9].

It is now recognized that eosinophilic inflammation is the hallmark of asthma [11], and that the levels of ECP in BAL fluid are elevated in asthmatic patients, even under steady state conditions [26]. The present study is unique in that BAL was performed during the course of asthma exacerbation. Unexpectedly, the extent of the increase in BAL ECP and eosinophil numbers was not marked, but similar to that observed during the stable period by other investigators [27, 28]. ECP is a highly charged protein and may have been lost in the collection or concentration stages. The validity of the collection or concentration procedures should have been checked to rule out this possibility. Another factor that should be considered is systemic corticosteroid treatment as an absolute medical indication for acute asthma exacerbations. Corticosteroids can reduce the level of BAL eosinophil numbers and degranulation [29]. There is no data available on the kinetics of inflammatory changes in BAL fluid after treatment with systemic corticosteroids in an acute exacerbation of asthma. Pizzichini et al. [29] have showed that treatment with prednisone improved the forced expiratory volume in 1 s and blood inflammatory indices within a day, but resulted in only gradual improvement in sputum indices between day 2 and day 7. We performed bronchoscopy with BAL on the 4th day after admission to minimize the procedural risks, including hypoxemia, during the early exacerbation phases of acute asthma. This may partly explain our finding that BAL eosinophilia in our asthmatic subjects were comparable with that seen during asymptomatic period by other investigators [27, 28]. However, of note, eosinophil numbers in asthma subjects were still higher compared with bronchiolitis and control subjects.

We did not find any increases in the IL-8 levels, in contrast to the previous two studies that assessed induced sputum parameters in children with acute asthma [30, 31]. Another study reported no difference in BAL IL-8 levels between normal and asthmatic subjects [32]. Viruses are an important cause of asthma exacerbations in children, and are potent inducers of a neutrophil response. It has been postulated that IL-8 released from macrophages and/or epithelial cells infected with the viral particles, may have a chemoattractant

effect on neutrophils [33]. The lack of elevated IL-8 levels in the current study may be attributed to the exclusion of asthmatics with suspected or proven viral etiologies or to the use of corticosteroids during the course of asthma exacerbation. Corticosteroids have been demonstrated to inhibit airway epithelial cell IL-8 secretion *in vitro* [34]. However, this effect has not been clearly demonstrated *in vivo*. For example, sputum IL-8 concentrations have been shown to remain significantly elevated in severe persistent asthma, despite high-dose corticosteroid treatment [18]. In the present study, the relatively low levels of IL-8 found in the three acute asthmatics who underwent BAL prior to corticosteroid administration, also argues against the importance of corticosteroids in terms of reducing the IL-8 level *in vivo*.

Several studies have been undertaken on eosinophil activation in acute bronchiolitis. The finding that leukotriene C₄ was elevated in the nasopharyngeal secretions of children with RSV bronchiolitis [35] was regarded as evidence of eosinophil activation, since leukotriene C₄ is the major leukotriene secreted by eosinophils [36]. Another study found that levels of ECP in the nasopharyngeal secretions of infants with RSV bronchiolitis were higher than in samples from infants with RSV upper respiratory tract illness only [37]. However, there are contrasting findings by others [38] who showed that the levels were not different. Also, data obtained from these studies are difficult to interpret, because they do not necessarily reflect events in the lower respiratory tract. In our study, ECP levels in BAL fluid from children with acute bronchiolitis did not differ from those of the control children.

In vitro studies have shown that RSV infection of airways stimulates alveolar macrophage [39] and epithelial cells [40] to release IL-8. One group reported elevated plasma IL-8 levels in infants with RSV bronchiolitis [41]. Another study showed that IL-8 levels are significantly elevated in the nasal secretions of infants with acute bronchiolitis [42]. Our results indicate that IL-8 is released *in vivo* within the lower respiratory tract of infants with RSV bronchiolitis and that it is positively correlated with neutrophil percentages in the BAL fluid. This suggests that the accumulation of neutrophils in the lower airway may be mediated by IL-8, and that IL-8 is likely to play a major role in promoting the intense inflammatory process evident in the airways of infants with acute bronchiolitis.

One of the shortcomings of our study is that our control group for bronchiolitis subjects is inadequately matched for age. In a previous study [43], a weak but significant correlation was found between age and ECP in respiratory secretions, i.e., older patients were found to have higher ECP values than younger patients. Thus, the lack of a significant difference in the ECP levels of the bronchiolitis group and the control group might reflect an increased ECP level in acute bronchiolitis. On the other hand, serum IL-8 concentration is reported to decrease with age [44], probably because younger children experience more viral infections than are older children. We cannot therefore exclude the possibility that the elevated IL-8 levels in the bronchiolitis group might be ascribed to their younger age. Further studies that utilize age-matched controls for the bronchiolitis group are needed to resolve this issue.

In summary, our BAL fluid analysis shows that ECP levels in children with acute asthma and IL-8 levels in children with acute bronchiolitis are elevated. These differences in the biomarker profiles, together with different inflammatory cell patterns, suggest that the nature of the inflammatory process within the respiratory tract is dissimilar in these two diseases.

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