

Eosinophil cationic protein in nasopharyngeal secretions and serum of infants infected with respiratory syncytial virus

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Eosinophil cationic protein (ECP) was assayed in nasopharyngeal secretion (NPS) and serum from 42 infants, hospitalized with acute lower respiratory infection, in El Salvador and the results analyzed in relation to etiology of the infection. ECP concentrations were high in NPS, at an average 50 times higher than those found in serum. Exceedingly high levels of ECP ($>1000 \mu\text{g/L}$) were found more frequently in wheezing than in non-wheezing children (30% vs 7%) and, accordingly, were more commonly found in children hospitalized with bronchiolitis than in those with pneumonia. Excessive levels were significantly more common in girls than in boys. Of the 42 cases, 28 were found to be caused by respiratory syncytial virus (RSV) subgroup A, and 3 by RSV-B, by means of detection of RSV antigen in nasopharyngeal cells. ECP serum levels were moderately elevated during the acute phase of the respiratory infection and increased slightly but significantly, in cases with RSV antigen-positive bronchiolitis, but not in those with pneumonia. The ECP levels in NPS from patients in Sweden who, by antigen detection in NPS cells, were diagnosed as either RSV or para-influenza 3 infection or none of these, were similar. These results indicate that elevation of ECP in NPS is associated with acute lower respiratory infection in general, but particularly pronounced in cases of bronchiolitis. Elevation of ECP is not an exclusive consequence of RSV infection, but may occur to an equal extent in infections caused by other agents. Girls generally seem to be less prone than boys to developing airway obstruction and may, therefore, acquire severe bronchiolitis only when large amounts of inflammatory mediators, such as ECP, accumulate in the airways.

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Upon stimulation, eosinophil granulocytes secrete several toxic proteins, one of which is the eosinophil cationic protein (ECP). ECP is a highly basic and potent cytotoxic single-chain zinc-containing protein present in eosinophil granules (1). This protein has been suggested to be involved in the defence against bacteria and parasites, and in the pathogenesis of allergic, and other inflammatory diseases, as well as in bronchiolitis related to respiratory syncytial virus (RSV) infections (1–3). RSV is the major cause of bronchiolitis and pneumonia in small children (4, 5) and bronchiolitis in infancy has been suggested to be associated with the development of reactive airway disease later in life (6–9).

In chronic asthma several studies have shown presence of ECP as well as leukotrienes, and histamine in areas where sloughing of the epithelium has occurred, as a result of an ongoing inflammatory process (10–12). In bronchiolitis high concentrations of inflammatory mediators have been found (2, 13–16) suggesting that they may be important in the pathogenesis of obstructive airway disease.

The aim of the present study was to determine the level of ECP in nasopharyngeal secretions from hospitalized children suffering from bronchiolitis or pneumonia caused by RSV and to compare findings obtained in a hospital in El Salvador with those in children from Sweden, suffering from acute lower

respiratory infection caused by RSV, para-influenza type 3 or other agents.

Material and methods

Forty-two acute blood samples, and acute nasopharyngeal specimens (NPS) were obtained from consecutive cases of less than one year-old children hospitalized with acute lower respiratory infection (ALRI) at the Children's Bloom Hospital in San Salvador, El Salvador. The time of specimen collection started on December 12, 1991 and ended January 31, 1992. The RSV epidemic in El Salvador this season apparently started at the end of October and lasted until the end of January (personal information from the physicians).

All the hospitalized children studied were diagnosed by clinicians as having either severe bronchiolitis or pneumonia. Bronchiolitis was defined by chest radiographs revealing hyperinflation with or without atelectasis, together with wheezing on auscultation. Pneumonia was defined by radiographic evidence of consolidated pulmonary infiltrates, determined by a radiologist. The assessment of the overall severity of the disease was made on the basis of signs, symptoms and chest x-rays. The mean age of these children was 4.6 months (range 0 to 12 months); 64.3% (27 patients) were males and 35.7% (15 patients) were females. Thirty-two infants (19 boys and 13 girls) had bronchiolitis and 10 (8 boys and 2 girls) had pneumonia. None of the children were treated with ribavirin or any other antiviral drug.

Acute NPS and serum samples were collected a maximum of 48 h after admission to hospital. One NPS sample only was taken from each individual. Sera from convalescence were obtained from 25 of the 42 patients, four weeks (± 3 days) after sampling of the acute sera. All the patients were living under poor socio-economical conditions.

Thirty-six NPS were provided by the Central Microbiological Laboratory, Stockholm, Sweden, from less than two year-old children with ALRI in different hospitals in Stockholm, during January 7 to June 6, 1992. Their mean age was 6 months (range 0 to 24 months). Since these samples were not taken as a part of a prospective study, no detailed information about the children's clinical condition, severity of disease etc, was available, and the material was included in the study for the sole reason of determining NPS ECP levels in ALRI caused by different types of agents. Of the patients 10 were RSV-positive, 10 were para-influenza type 3-positive and 16 were negative when tested by direct immunofluorescence technique and virus culture. Serum samples, collected at routine visits to two health units in

San Salvador from healthy children were used as controls.

Specimen collection in El Salvador

Blood samples were obtained from the antecubital vein with a sterile syringe. Between 1.5 and 2 ml was collected from <6 month-old infants, and approximately 3 or 4 ml from older children. The blood samples were placed in sterile centrifuge tubes without any preservatives and, after coagulation, within 1 h after collection, they were centrifuged twice at 2000 rpm for 10 min. The sera were transferred to Nunc cryotubes with 1.5 ml or less in each, and stored at -20°C until used.

Nasopharyngeal samples were obtained by suctioning into a soft sterile catheter (Vygon company, France) introduced through the nostril and connected to a sterile mucous trap. The secretions were rinsed using 5 ml of sterile Hank's balanced salt solution containing 2% fetal calf serum with penicillin (500 U/ml), streptomycin (500 $\mu\text{g/ml}$) and nystatine (100 U/ml). Following transportation to the laboratory of the chilled diluted aspirate (about 6 ml), the cells were pelleted by centrifugation at 1500 rpm at 4°C for 10 min and the supernatant collected and stored at -20°C . The protein concentration in the supernatants was not determined. The cells were then washed with phosphate buffered saline (PBS), pH 7.2 with 0.005% NaN_3 , spotted onto a microscopy slide (six drops from each sample on the slide), fixed with cold acetone for 10 min and finally stored at -20°C for further use.

All sera and supernatants of the nasopharyngeal samples were stored for 10–12 months at -20°C in El Salvador, then transported in dry ice to Sweden where they were stored at -20°C for another 3 months before being tested.

Immunofluorescence

RSV antigen in nasopharyngeal secretions was determined by indirect immunofluorescence technique and five monoclonal antibodies (Mabs) specifying 5 epitopes of RSV were used on cell smears to determine RSV subgroup identity. The monoclonal antibodies used included 3 antibodies directed against the G protein specific for 3 epitopes and 2 antibodies against the NP protein. These Mabs were kindly provided by Britt Åkerlind, Karolinska Institute, Stockholm, Sweden.

The Mabs, diluted 1:50, were incubated with the fixed nasopharyngeal cells at 37°C for 30 min in humidity chamber. The slides were then washed 5 times in PBS, pH 7.2, and 25 μl drops of conjugated rabbit FITC anti-mouse immunoglobulin (DAKO, Copenhagen, Denmark) diluted 1:20 were added.

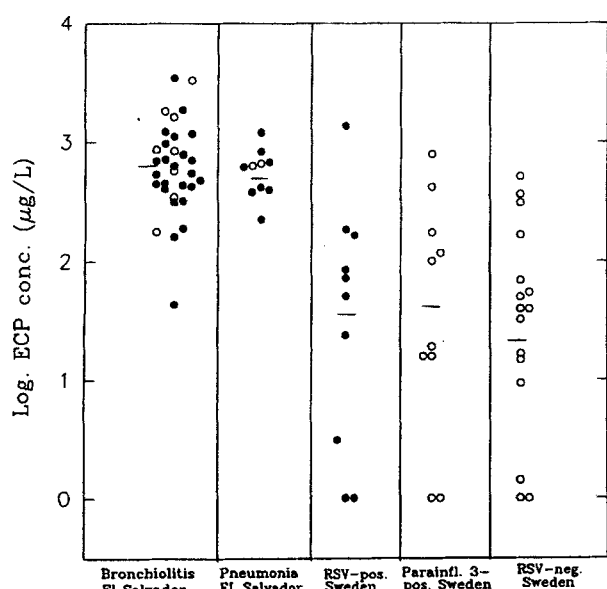


Fig. 1. ECP in nasopharyngeal secretions. Filled circles represent RSV-positive cases and empty circles RSV-negative cases. Horizontal bars represent median values.

The slides were incubated at 37°C for 30 min and washed as above. Evan's blue (1:30000) was used for counterstaining. The slides were mounted in buffered glycerol (90% pure glycerol mixed with 10% PBS, pH 7.2–7.5) and analysed in a Leitz fluorescence microscope.

In NPS from Sweden, direct immunofluorescence technique was used on cell smears of nasopharyngeal aspirate for detection of RSV at the Central Microbiological Laboratory of Stockholm County Council, Stockholm, Sweden (SMCL) using a directly FITC-conjugated subgroup non-specific, anti-RSV-monoclonal antibody from Ortho Diagnostic Systems Inc., NY, USA.

Specimens from Sweden

The NPS from patients in Sweden were diluted in sterile sodium chloride. The final volume of the

material was 2 ml with approximate dilution of the NPS of 1: 2 or 1: 4, and the specimens were placed on ice for transportation to the laboratory.

Virus isolation

Virus isolation attempts were performed on the NPS with the purpose of detecting RSV, influenza A and B viruses, para-influenza and adenoviruses. These samples were inoculated into tubes containing green monkey kidney (GMK) cells, rhabdomyosarcoma (Rd) cells, canine kidney (MDCK) cells, Hela cells and Ma 104 cells with 0.5 µg/ml of trypsin.

Determination of ECP

A total of 50 µl of each sample was used and the ECP concentration determined by means of a competitive radioimmunoassay, performed as described by the manufacturer (Pharmacia ECP RIA, Kabi Pharmacia Diagnostics, Uppsala, Sweden). The assay for ECP has shown no cross-reactivity with other eosinophil proteins or neutrophil proteins (2). Levels of ECP in nasopharyngeal samples were adjusted for the volume of sample recovered, and expressed as micrograms per liter of fluid.

Statistical analyses

Student's t-test for comparison of means of independent samples and paired samples were used to test the different groups. Wilcoxon's signed rank test was used to compare paired serum samples. Linear regression and correlation was used for assaying correlations between ECP levels in NPS and serum and the Quick test – chi-square was used for comparison of proportions of positive samples.

Results

ECP in child nasopharyngeal secretions (El Salvador)

As shown in Fig. 1 the levels of ECP in NPS from children in El Salvador were generally very high

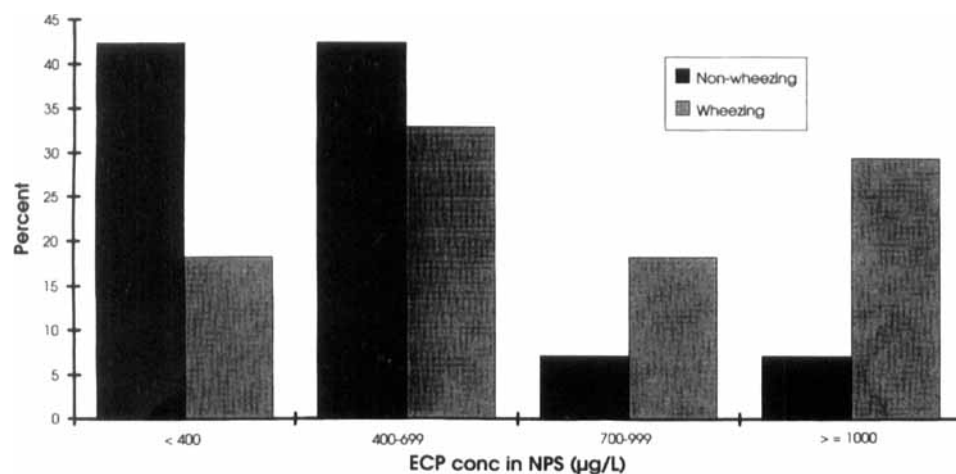


Fig. 2. Frequency distribution of ECP levels in NPS of 42 children in relation to wheezing.

Table 1. ECP levels in NPS from boys and girls.

Patients	n	Median values	No. exceeding 1000 $\mu\text{g/L}$
Bronchiolitis			
boys	19	461**	2**
girls	13	876	6
Pneumonia			
boys	8	525	0
girls	2	912	1
All patients			
boys	27	461**	2***
girls	15	876	7

Differences between boys and girls regarding median values and proportions with exceedingly high ECP levels calculated by Wilcoxon's test and chi-square test respectively. ** $p < 0.01$; *** $p < 0.001$.

(median 625, range 44–3,440 $\mu\text{g/L}$). Although the median ECP values in patients with bronchiolitis ($n = 32$) or pneumonia ($n = 10$) were not significantly different (631 and 619 $\mu\text{g/L}$ respectively) a higher proportion of the cases with bronchiolitis had exceedingly high ECP values ($> 1000 \mu\text{g/L}$) than those with pneumonia (8/32 vs 1/10). Similarly, as seen in Fig. 2, if the single parameter of wheezing was considered, wheezing children had ECP levels above 1000 $\mu\text{g/L}$ more frequently than non-wheezing children (8/27 vs 1/14, $0.10 > p > 0.05$). The median ECP levels in RSV-positive cases were not significantly different from those in RSV-negative children (563 vs 659 $\mu\text{g/L}$) and neither was there any significant difference in the proportion of children with levels above 1000 $\mu\text{g/L}$ between these groups (7/32 vs 2/10).

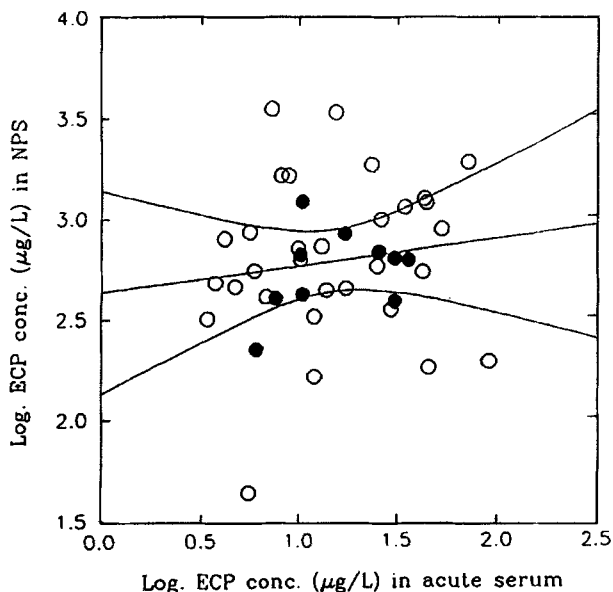


Fig. 3. Relationship between NPS and acute serum levels of ECP. Filled circles represent cases with pneumonia and empty circles cases with bronchiolitis. Curves indicate 99% confidence interval.

The levels of ECP in the three children infected with RSV-B were 324, 461 and 545 $\mu\text{g/L}$, respectively. As seen in Table 1 NPS levels of ECP exceeding $> 1000 \mu\text{g/L}$ were found more often in girls than boys in the total material (7/15 vs 2/27; $p < 0.001$), as well as in the cases with bronchiolitis (6/13 vs 2/19; $p < 0.01$). Also, the median levels of ECP were higher in girls than in boys in the total material, as well as in the cases with bronchiolitis (876 vs 461 $\mu\text{g/L}$ for both comparisons, $p < 0.01$ and $p < 0.001$, respectively). A tendency toward higher ECP levels in older children was noted; thus the mean age of children having levels below 500 $\mu\text{g/L}$ was 4.1 months and in those with levels exceeding 1000 $\mu\text{g/L}$ it was 5.7 months. Although the average age of the girls (5.6 months) was higher than that of the boys (4.0 months) a possible age dependency of ECP levels could not account for the observed gender difference. Thus, the median ECP level in girls (876 $\mu\text{g/L}$) was still substantially higher than in strictly age-matched boys (534 $\mu\text{g/L}$), and the proportion of cases with ECP levels exceeding 1000 $\mu\text{g/L}$ was significantly higher in the girls than in the age-matched boys ($p < 0.05$).

ECP in child nasopharyngeal secretions (Sweden)

For unclear reasons the levels of ECP in NPS samples from Sweden were much lower than those encountered in samples from El Salvador.

As shown in Fig. 1 the median levels of ECP, however, were similar in NPS samples obtained from patients with RSV-infection, para-influenza 3 infection and in those obtained from patients with suspected, but not confirmed viral infection (median values 62, 64 and 48 $\mu\text{g/L}$, respectively). A value exceeding 1000 $\mu\text{g/L}$ was found in one child only. This child belonged to the RSV-positive group.

ECP levels in acute and convalescent phase sera (El Salvador)

Serum ECP levels in all groups of children with ALRI were increased as compared to levels found in uninfected children from El Salvador (Table 2). Levels in acute sera were slightly lower than those in convalescent serum samples (mean 14.7 and 20.6 $\mu\text{g/L}$, respectively).

A significant rise ($p < 0.05$) in ECP levels was seen in RSV-positive cases with bronchiolitis (Table 2). A rise above 16 $\mu\text{g/L}$, which is the upper normal limit for serum ECP, given by the kit manufacturer, was seen in 45% of all acute phase sera and in 70% of the convalescent phase sera. There was no significant correlation between ECP levels in NPS and serum, neither in cases with pneumonia ($r = 0.32$,

Table 2. ECP levels in acute and convalescent sera from hospitalized children and in healthy controls.

Serum	RSV	n	Geom. mean ($\mu\text{g/L}$)	Range	No. of sera with ECP above 16 $\mu\text{g/L}$
Br. acute	+	13	14.1	4.2– 44.6	5
Br. conv.	+	13	22.3*	6.9–108.0	10
Br. acute	–	4	34.6	23.5– 53.2	4
Br. conv.	–	4	26.0	7.6– 72.9	3
Pn. acute	+	7	15.8	6.0– 36.7	4
Pn. conv.	+	7	17.8	9.2– 28.8	4
Br. + Pn. acute	+	20	14.7	4.2– 44.6	9
Br. + Pn. conv.	+	20	20.6	6.9–108.0	14
Br. acute	+ & –	17	17.4	4.2– 53.2	9
Br. conv.	+ & –	17	23.1	6.9–108.0	13
Controls	–	34	11.1	5.5– 16.6	0

* Significant increase ($p < 0.05$) between acute and convalescent sample.

$p=0.36$) nor in those with bronchiolitis ($r=0.10$, $p=0.39$) (Fig. 3).

Discussion

Bronchiolitis, or wheezy bronchitis, is a disease of infancy that primarily is caused by respiratory syncytial virus (RSV) subgroups A or B, although other agents, such as para-influenza viruses occasionally may be the cause of this syndrome (17). In some studies (18, 19), but not in others (20, 21), RSV-A has been found to give rise to more severe disease than RSV-B. In the present study of acute lower respiratory infection in hospitalized infants in El Salvador, RSV-A greatly dominated over RSV-B as the etiological agent of bronchiolitis. Nevertheless, two of the 42 cases were severe enough to require intensive care; these cases were among 3 cases caused by RSV-B. Thus, although based on a limited number of cases, the present results gave no support to the previously made claim that RSV-A causes a more severe infection than RSV-B (18, 19).

Bronchiolitis, caused by RSV, has previously been shown to be associated with pronounced inflammatory reaction in the airways, involving release of inflammatory mediators like the leukotrienes LTB_4 and LTC_4 (13) as well as eosinophil cationic protein (ECP), which is a toxic product of activated eosinophils. In accordance with the results of Garofalo et al. (2), we found high levels of ECP in infants with bronchiolitis. In fact, the levels found by us were substantially higher than those measured by Garofalo et al. (2) and also much higher in children from El Salvador than in children from Sweden. The reasons for these discrepancies are unclear but may be due to differences regarding handling of specimens or to varying severity of disease between the different population studied. It should be noted, however, that ECP levels similar to those found by us in NPS of children from El Salvador have been

found by others in nasal secretions of patients with allergic rhinitis (22).

In agreement with previous results (2), we found higher levels of ECP in NPS from wheezing than from non-wheezing children. Yet, substantial elevations were observed also in some non-wheezing children, and, conversely, some children with bronchiolitis had only moderately increased ECP levels. Thus for instance, two boys with RSV-B-induced bronchiolitis requiring intensive care did not have excessive levels of ECP. These findings suggest that constitutional factors like e.g. airway size are decisive in determining whether a child will respond to a given concentration of ECP (or some other substance(s) rising in parallel with ECP) by developing a clinical picture of bronchiolitis. If this assumption is correct it could be predicted that boys, who have smaller airways than girls (23, 24) would develop bronchiolitis easier than girls, in response to moderate concentrations of ECP, and also, according to the same assumption, older infants, if requiring hospitalization, would have higher NPS levels of ECP than younger children. In fact, both predictions were found to be true; thus the median levels of ECP were almost twice as high in girls as in boys with bronchiolitis and the proportion of cases with excessive ECP levels ($> 1000 \mu\text{g/L}$) was higher in girls than in boys (7/15 vs 2/27; $p < 0.001$). Likewise, there was a tendency toward higher age in the children having high ECP levels, than in those having low levels.

Raised ECP levels in NPS have previously been observed mainly in bronchiolitis caused by RSV (2). We found that also bronchiolitis caused by other agents, such as para-influenza 3, may result in elevation of ECP to a similar degree as that observed in RSV-infections. It seems reasonable to assume that bronchiolitis may result from an inflammatory reaction in the bronchioli caused by a variety of agents. Release of inflammatory mediators, such as histamine, has been observed *in vitro* following stim-

ulation with RSV, as well as para-influenza 1 and 3 viruses (25).

The serum ECP levels were in this study moderately increased during the acute phase of the disease in most patients, and a significant further rise from acute to convalescent phase was observed in RSV-infected infants with bronchiolitis. A delayed rise of ECP levels would be expected to occur if, as previously claimed (16, 26), RSV-specific IgE antibodies are involved in the pathogenesis of bronchiolitis. IgE antibodies, which are produced subsequent to the acute infection, would be assumed to sensitize mast cells which in turn may recruit and activate eosinophils to secrete products like ECP (27). Since, conversely, eosinophil secretion products can trigger mast cell secretion, a vicious circle may be formed, resulting in a perpetuation of a bronchial hyperreactivity state. In this context, it is interesting that experimental para-influenza type 1 bronchiolitis in newborn rats results in a transient respiratory infection followed by a progressively severe pulmonary inflammatory disease associated with e.g. eosinophil degranulation and airway hyperreactivity (28). Our finding of increasing serum levels of ECP following an acute bronchiolitis in infants suggests that similar events may occur in humans. An infection caused by a respiratory virus may thus cause a prolonged inflammatory reaction in the airways which might predispose the child for further episodes of wheezing, and possibly, for later development of asthma.

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