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Original article

Eosinophil cationic protein and eosinophil protein X in the nasal lavage of children during the first 4 weeks of life

Eosinophil cationic protein (ECP) and eosinophil protein X (EPX) are well established as markers of eosinophil activation. We analyzed ECP and EPX concentrations in nasal lavage fluids (NALF) of 378 neonates during their first 4 weeks of life. Inclusion criteria were a positive history of parental allergy and a positive skin prick test or specific IgE (RAST class ≥ 2) against at least one out of a panel of common aeroallergens in one or both parents. Twenty-four infants with no history of parental allergy were used as controls. A volume of 2 ml of 0.9% saline was instilled into each nostril and immediately recovered by a suction device. ECP and EPX were analyzed by radioimmunoassay. In 65 samples of three consecutive lavages, EPX was detected in nine samples (13.8%) in the control group, whereas it was detected in 197/360 samples (54.7%) in the study population. The corresponding figures for ECP were 17/65 (26.2%) in the control group and 173/365 (47.4%) in the study group. Both proteins showed a skewed distribution (median/5–95th percentiles for ECP: 0 μg/l [0–69.4] and EPX: 6.6 μg/l [0–73.2]). The differences between the control group and the study group were statistically significant, regardless of the allergic disease of the parents. In children of allergic parents, activation proteins of the eosinophil granulocyte are released on the nasal mucosal surface in about 50% of the studied population at the age of 4 weeks. This early onset of eosinophil activation in the nasal respiratory epithelium may reflect a genetic predisposition to allergy or early exposure to allergens.

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Allergic diseases seem to be partially genetically determined (1). However, as specific genetic markers are not available, prediction of allergic diseases on a genetic basis seems to be impossible. Since early intervention may modulate the natural course of atopic disease, the availability of predictive markers is of considerable interest (2, 3). Until now, it seemed difficult to define subgroups as potential candidates for secondary prevention. Family history of atopic disease and cord-blood IgE have been used as predictors of allergic diseases in children (4). However, their predictive capacity was shown to be rather poor, so that the majority of atopic manifestations occur in infants with no demonstrable risk at birth (4, 5). In recent years, increased serum levels of eosinophil granule proteins (eosinophil cationic protein [ECP], eosinophil protein X [EPX], eosinophil peroxidase [EPO], and major basic protein [MBP]), despite normal eosinophil numbers, have been reported in atopic children (6, 7). Elevated levels of ECP or EPX have been demonstrated in bronchial asthma, atopic dermatitis, and allergic rhinitis (8–10). These proteins are widely accepted as specific markers of eosinophil activation. They can be determined in bronchoalveolar lavage, sputum, serum, and urine (11). Recently, nasal lavage has been used to study airway inflammation in asthmatics, a method that is well tolerated by children (12–15). As nasal mucosal histology is similar to that in the lower airways, and inflammatory processes in the nasal air passages may reflect those in the lower airways, concentrations of eosinophil proteins in nasal lavage fluid (NALF) might provide more accurate information than those in sera or urine about eosinophil activation in the lung (12). To determine whether eosinophil proteins are released on the nasal mucosa surface at a very early stage of life, we analyzed ECP concentrations in the NALF of 378 neonates during their first 4 weeks of life. Our objective was to determine whether eosinophils are activated on the nasal mucosa of neonates and whether there is a relation to atopic family history.

Material and methods

Study design and subjects

All of the children participated in an international multicenter study (Study on the Prevention of Allergy in Children in Europe (SPACE, Biomed II program of the EC; grant no. PL95-1211) dealing with the prevention of allergy by dust-mite-avoidance measures.

Mothers were approached either at routine visits to two obstetric wards in Vienna (Austria) (Allgemeines Krankenhaus der Stadt Wien [AKH], Sozialmedizinisches Zentrum Ost [SMZ-Ost]) during pregnancy, or shortly after birth at the children's departments of the respective clinics. Screening questionnaires for symptoms associated with allergic diseases were distributed. If a history of allergic rhinitis, hay fever, bronchial asthma, or eczema was reported by any parent, the skin prick test (SPT) (ALK Scherax, Hamburg/Germany) or serum IgE measurement (CAP system, Upjohn & Pharmacia) was performed. Families were included in cases of positive allergy history and positive allergy tests (*Dermatophagoides pteronyssinus*, *D. farinae*, birch, ryegrass, ragweed, and cat) in any parent. This report covers the period April 1997 to June 1998. A first cross-sectional survey was carried out in 397 neonates (age [mean 20.6 days, SD 7.1]). They were visited at home to perform nasal lavages and to implement allergy-prevention measures. The intervention consisted of a specially designed booklet and a mattress cover impermeable to house-dust mites for the children and all other beds that they might use (data will be presented elsewhere). Data on parental education, smoking habits, housing modalities, and pets in the home were gathered by a questionnaire. Informed consent was obtained from the parents before all measurements, and the study protocol was approved by the Vienna University's ethics committee and the ethics committee of the SMZ-Ost.

Twenty-four children with no history of parental allergy (age [mean 25.8 days, SD 31.7]) were used as controls. These children were recruited from the outpatient clinic of the University Children's Hospital in Vienna. Inclusion criteria were a negative parental history of allergic disease and no current respiratory symptoms or febrile illness at the time of lavage.

Allergy testing

The SPT was carried out by trained members of the staff under the supervision of a physician, who used the same equipment and technique each time. The allergens (*D. pteronyssinus* (D.pt.), *D. farinae* (D.f.), cat, birch, ryegrass, and ragweed; ALK Scherax, Hamburg, Germany; concentration: 10 histamine equivalent in prick testing) and the negative (sodium chloride 9 g/l) and positive controls (histamine hydrochloride 10 mg/ml) were applied to the forearm with an ALK prick needle (ALK Scherax, Hamburg, Germany). The wheal reactions were marked with a pen, and the outline was transferred to paper with a transparent strip. Then the largest and the perpendicular diameter of each wheal reaction were measured with a transparent ruler, and the arithmetic mean was calculated. A positive test required a wheal diameter at least 3 mm larger than the negative control. Sensitization to a mite allergen required a positive test to D.pt. or D.f. RAST was done with the same panel of aeroallergens. Families were included in case of a positive allergy history and positive allergy tests (SPT or specific IgE [RAST class ≥ 2]) in any parent.

Nasal lavage fluid

Families were visited when the child was healthy, as judged by the parents within the first 4 weeks of life. Nasal lavages were performed at the children's homes. On inspection of the nasal lavage fluid, children were defined as being free of rhinitis, having serous rhinitis (watery secretions), or having purulent rhinitis (thick, yellowish secretions).

Nasal lavage was obtained by instilling with a needleless syringe 2 ml of 37°C physiologic saline solution into each nostril with the baby lying supine. Wash fluid and secretions were immediately aspirated into a sterile specimen trap (a combination of a tracheal suction set (Fa.Dahlhausen) and a 30-cm length of suction catheter (Fa.Uno) attached to a portable suction device (Atmos LC-16, Fa.Draeger).

Each nostril was washed. The washes resulted in volumes of 502 µl (SD $\pm$ 308 µl) of fluid and secretions. The samples were processed in a standardized way. Samples were weighed and diluted 1:1 with dithiothreitol (Cleland's Reagent, Fa.Bio-Rad). They were centrifuged at 1454 *g* for 10 min at room temperature. Cells were discarded, and the supernatants were stored at -30° until analysis. Supernatants were used for analyses of eosinophil proteins. Results are given in µg/l after correction for the dilution. Eight medical students, thoroughly trained in the procedure, performed all the lavages.

Lavage fluid measurements of ECP and EPX

Serum ECP and EPX concentrations were determined by a specific and sensitive double-antibody radioimmunoassay (Pharmacia Upjohn AB, Uppsala, Sweden). ECP and EPX in the sample compete with a fixed amount of ^{125}I-labeled ECP/EPX for the binding sites of specific antibodies (17). The intra-assay coefficient of variation was less than 3%, and the interassay coefficient of variation was below 11% for all assays. Analyses were done at the same time.

Statistical analysis

Statistical analysis was done by the SAS package, Version 6.11. Results are presented as median (5–95th percentile) unless stated otherwise. Differences between the groups were examined by the Wilcoxon rank-sum test. Conventional multiple linear regression was used to investigate the association of ECP and EPX with potential covariates in the sample. For regressional analyses, data were first log transformed, and if ECP/EPX was not detectable, values were treated as dummy variables. The level of significance was considered as being at the fifth percentile ($P<0.05$).

Results

From the target population (average number of birth/year: SMZ-Ost: 2000, AKH: 2000), screening questionnaires were distributed to a total of 4309 parents (1261 SMZ-Ost and 3048 AKH) between March 1997 and June 1998. Questionnaires were distributed simultaneously in the obstetric wards and the neonate wards of the three hospitals. Of these, 4159 were returned. A positive answer to any of the screening questions was given in 24.7% of replies ($n=1227$). History of symptoms associated with allergic disease was confirmed by a positive SPT or specific IgE (RAST class ≥ 2) in 553 parents. Of these, a total of 408 parents consented to participate in the study. After birth, 11 children did not fulfil the inclusion criteria (reasons: low birth weight, admission to the intensive care unit for more than 7 days, and twins). Hence, a total of 397 children entered the study. The average birth weight of the children was 3370 g (SD 440 g).

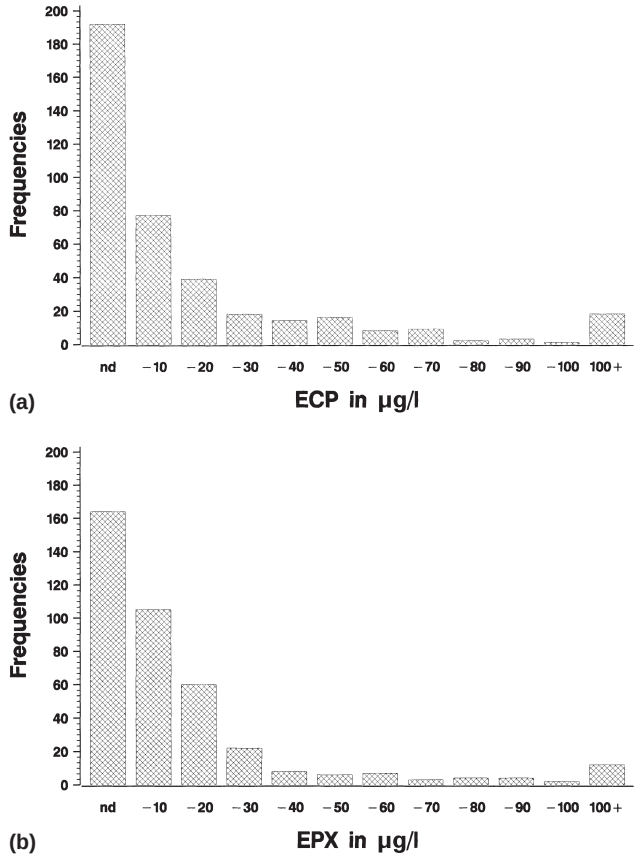

Figure 1. Distribution of frequencies of ECP (a) and EPX (b) concentrations in infants with positive parental allergic history in nasal lavage fluid. ECP: eosinophil cationic protein; EPX: eosinophil protein X; nd: not detectable.

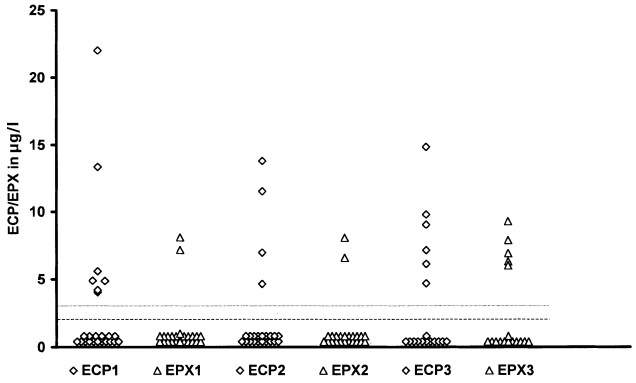

Figure 2. Concentrations of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) in infants with no parental allergic history in nasal lavage fluid. Nasal lavages were performed in 24 children (ECP1/EPX1). Of these, in 23 infants, lavages were done second time after 1 h (ECP2/EPX2) and in 18 again after 72 h (ECP3/EPX3). Detection limit for ECP (——) was 2 µg/L, and for EPX (.....) 3 µg/L.

Nasal lavage

Nasal lavage was performed in 378 of 397 children (95.2%). At the home visit, 318 children were free of any nasal discharge, 69 had some serous nasal secretions on inspection, and 10 had overt purulent rhinitis. None of the children had signs of eczema or respiratory symptoms. Average recovery was 502 µl (SD 311 µl). The variability of recovery between observers ($n=8$) was 6.9%. Data from the 10 children with purulent

Table 1. Characteristics of infants with positive parental allergic history and concentrations of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) obtained in nasal lavages 4 weeks after birth

		n/%	ECP (µg/l)	EPX (µg/l)
Sex*	M	171/46.5	18.7 ± 55.9	18.5 ± 50.6
	F	197/53.5	19.2 ± 52.7	24.1 ± 124.4
Smoking during pregnancy*	Yes	71/19.3	19.2 ± 39.8	16.3 ± 33.5
	No	297/80.7	19.0 ± 57.2	22.8 ± 107.6
Passive smoking*	Yes	78/21.3	19.9 ± 57.9	12.8 ± 30.4
	No	286/78.7	16.0 ± 37.6	23.8 ± 108.9
Education*	High	225/61.1	24.4 ± 67.3	28.6 ± 123.2
	Low	143/38.9	10.7 ± 18.9	10.3 ± 20.1
Number of persons in household*	≤ Three	217/59.0	16.3 ± 60.7	23.5 ± 124.6
	Four	117/31.8	24.9 ± 45.8	19.9 ± 32.8
	≥ Five	34/9.2	16.2 ± 31.1	15.2 ± 34.7

*Results are presented as mean ± standard deviation; children with purulent rhinitis have been excluded.

Table 2. Concentrations of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) in infants subdivided into groups according to parental allergic history and in controls

	ECP (μg/l)	*P* value**	EPX (μg/l)	*P* value**
Control group*	2.5 ± 5.2	–	0.6 ± 2.2	–
Parental allergy*	19.0 ± 54.2	0.016	21.6 ± 97.6	< 0.0001
Parental asthma*	17.9 ± 49.9	NS	22.4 ± 89.0	< 0.0002
Parental hay fever*	19.9 ± 59.7	< 0.024	23.9 ± 109.6	< 0.0001
Parental rhinitis*	17.4 ± 47.7	= 0.024	21.2 ± 103.6	< 0.0001
Parental atopic dermatitis*	22.8 ± 54.7	= 0.02	27.0 ± 108.4	< 0.0001

*Results are presented as in Table 1.

rhinitis were not analyzed for the relationship between ECP and family history because of the likelihood of recent viral infection, their ECP values being more than 10-fold increased over the mean (232.6 μg/l *vs* 19.0 μg/l). ECP was detectable in 173 of 365 nasal lavages (47.4%) and showed a skewed distribution (Fig. 1A). The median (5–95th percentile) was 0 μg/l (0–69.38). There was no correlation between ECP concentration and recovery ($r = 0.06$; $P = 0.24$).

EPX was detectable in 197/360 samples (54.7%); the median (5–95th percentile) was 6.64 μg/l (0–73.15) and showed a skewed distribution (Fig. 1B). In 139 children, both ECP and EPX were detectable. The correlation ECP/EPX was $r = 0.819$ ($P < 0.0001$).

In the control group, 24 children were recruited, and nasal lavages were performed. In 23 of these infants, lavages were done a second time after 1 h and in 18 children again after 72 h. Results are shown in Fig. 2. Out of 65 samples, ECP was detectable in 17 samples (median [5–95th percentile] 0 μg/l [0–13.34]; 12 children), whereas EPX was detectable in nine samples (0 μg/l [0–7.16]; seven children).

A description of the study population is given in Table 1. Analysis was performed to investigate factors associated with increased ECP linear regression. Results show that there were no effects of observer, age, birth weight, smoking during pregnancy, social standards, size of household, sex, passive smoking, or season on ECP/EPX concentrations.

ECP/EPX concentrations in children with a history of parental allergy were significantly elevated compared to children with no history of parental allergy ($P < 0.0001$; Table 2). If one of the parents had allergic rhinitis ($P < 0.0001$), hay fever ($P < 0.0001$), or atopic dermatitis ($P < 0.0001$), ECP and EPX concentrations were significantly elevated compared to controls. If one of the parents had bronchial asthma, EPX levels were significantly increased ($P < 0.0002$), and ECP showed a strong trend (Table 2). Children with elevated ECP/EPX values did not have more risk factors (more than one parent atopic, both parents with asthma, hay fever, wheezing, atopic dermatitis, allergic rhinitis, or one or both parents with more than one symptom, higher social status, size of household, sex, or passive smoking) than children with no eosinophil proteins. Furthermore, we divided the children into one group with ECP/EPX higher than 20 μg and another group with a lower value. Again there was no demonstrable difference between the groups.

Discussion

The findings of this study demonstrated elevated levels of ECP and EPX in nasal lavage fluid in infants at 4 weeks of age with a high risk of becoming allergic. Out of 378 analyzed neonates, 231 demonstrated detectable concentrations of eosinophil proteins.

Until now, eosinophil activation has been measured most commonly in the sera of patients. However, a possible source of error in assessing inflammation by monitoring eosinophil activity is that products assessed in blood or urine are specific for eosinophilic activation, but not for eosinophilic airways inflammation. Since the respiratory epithelium is present in the nose, it has been assumed that inflammation processes in the lung may be reflected in the nose more accurately than in other body fluids. Recent studies in young children demonstrated higher levels of eosinophil proteins in the NALF of atopic patients or in patients with bronchial asthma or allergic rhinitis than in healthy controls (10–14, 17).

Nasal lavage is easy to obtain and well tolerated by the children. However, this method is difficult to standardize. The main issue in determining the "true" concentrations of a marker or mediator is the unknown dilution of the samples. In one of our previous studies, protein and albumin in the lavage fluid were measured in order to correct for dilution. However, in most of the samples, neither albumin nor protein was detectable in sufficient amounts. Therefore, the concentrations of ECP and EPX in the nasal lavage fluid could not be adjusted for albumin or total protein, as suggested elsewhere (18–20). This accounts for the large variability in concentrations. Hence, in spite of this methodological variability, our findings were highly significant. Eosinophilic activation occurs not only in allergic airway inflammation, but also in many other inflammatory disorders (7, 21). In children with probable viral disease, including purulent rhinitis, nasal lavages were not performed. Viral infection is known to elicit an inflammatory response in the upper airways (21). Hence we have excluded data from children who showed clinical signs of recent upper airways disease or febrile illness. Air pollutants have been shown to increase inflammatory markers on the nasal mucosa (22). In our data, passive smoking was not associated with nasal ECP/EPX. We do not assume that outdoor pollutants had affected these very young infants.

The expression of atopic diseases is a complex and multifactorial phenomenon, depending on an interaction of genetic factors and allergen exposure. There is

an increasing body of evidence that the immunologic changes leading to atopic diseases in later life are initiated during pregnancy. Raised cellular proliferation of cord-blood mononuclear cells in response to food allergens, as well as inhalant allergens, has been found in neonates with and without family history of atopy, indicating a previous antigen contact and an immune response (23). Hence, one possible explanation for the elevated inflammatory markers is related to intrauterine exposure to allergens.

No influences of passive smoking, smoking during pregnancy, sex, social status, or season on concentrations of eosinophil proteins were seen.

Whether early eosinophil activation may be interpreted as a possible onset of atopy remains subject to speculation. Recent studies have demonstrated that infants with high levels of eosinophil proteins during their first episode of wheezing illness subsequently developed recurrences of wheezing (24, 25). We do not know whether our results in respiratory tract secretions reflect the eosinophilic inflammation also seen in bronchial asthma. There are no studies which show that nasal lavage in atopic disease reflects a process in the lower airways. Therefore, we do not know whether elevated eosinophil products on the nasal mucosa are an indicator of lower airways inflammation or merely of a general disposition to allergies.

We conclude from this study that in children of allergic parents activation proteins of the eosinophil granulocyte are released on the nasal mucosa at the age of 4 weeks. Further follow-up of our cohort will determine whether the early onset of eosinophil activation in the nasal mucosa has a predictive value regarding the development of allergic diseases later in life.

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