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Short communication

Eosinophil-derived proteins in nasal lavage fluid of neonates of allergic parents and the development of respiratory symptoms during the first 6 months of life

Background: Eosinophilic airways inflammation forms the pathophysiologic basis for a proportion of children at risk of developing recurrent wheezing. Early preventive measures and/or anti-inflammatory treatment may be guided by the identification of such children.
Methods: We studied upper-airways inflammation by nasal lavage in a cohort of 397 infants within the first 4 weeks of life. They participated in an international multicenter study on the prevention of allergy in Europe (SPACE-Biomed II Program). A volume of 2 ml of prewarmed 0.9% saline was instilled into each nasal cavity and immediately re-collected by a suction device. The average recovery was 502 μl (SD: 311 μl). The concentrations of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) were determined by RIA analysis.
Results: ECP was detectable (>2 μg/l) in 47% of samples (173/365) and EPX (>3 μg/l) in 54.7% (197/360). Children with a doctor's diagnosis of a wheezy bronchitis within the first 6 months of life ($n=40$) had significantly higher ECP and EPX concentrations in the nasal lavage at 4 weeks of age (median ECP: 14 μg/l; 5–95th percentile: 0–122.4 μg/l) than children without such diagnosis (median ECP: 0 μg/l; 5–95th percentile: 0–86.6 μg/l; $P<0.05$). Corresponding figures for EPX were 12.14 μg/l (0–148.98 μg/l) *vs* 7.5 μg/l (0–81.46 μg/l; $P<0.05$). No associations between nasal ECP/EPX and the development of food allergy or eczema were observed.
Conclusions: Increased nasal ECP and EPX in the first 4 weeks of life are associated with wheezing in 6-month-old infants at increased risk of atopic disease. We suggest that this might be related to a general tendency for a T_h2 cytokine pattern in these young infants and subsequent trafficking of eosinophils into the nasal mucosa, or it might be a consequence of intrauterine allergen exposure.

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Recurrent wheezing is a common disorder in infancy. The phenotype is based on heterogeneous pathophysiologic mechanisms, of which at least two have been identified; namely, small airways disease and airway inflammation associated with the atopic trait (1). While the former has a favorable prognosis, with many children "outgrowing" their symptoms, the latter tends to persist. Early diagnosis and treatment have been shown to alter favorably the course of the disease (2). We have shown recently that young children with increased serum eosinophil cationic protein (ECP) at their first wheezing episode have an increased risk of developing recurrent wheezing (3). Hence, the detection of eosinophil airway inflammation might help to identify children at risk of obstructive airway disease. The nasal mucosa shares many properties with the lower airways, and it has been shown that, to some extent, changes in the lower airways are reflected in the upper airways mucosa (4, 5). Several techniques are available to sample the upper airways, of which nasal lavage is well suited for use even in small infants (6, 7). It is well tolerated by infants and their parents, it is hardly invasive, and it requires little equipment. Children with recurrent wheezing were shown to have higher concentrations of inflammatory markers in the nasal lavage then their asymptomatic peers (7, 8). Exposure to environmental hazards and virus can elicit similar inflammatory responses in the upper and lower airways (4, 5, 9). In a multicenter international study on the development of allergies in childhood, we followed a cohort of 397 neonates at risk of atopic disease (i.e., those with a parental history of allergic disease). We

tested the hypothesis that eosinophil upper airway inflammation, as measured by ECP and eosinophil protein X (EPX) in nasal lavage, is associated with the development of recurrent respiratory symptoms in this group of infants.

Material and methods

Study design and subjects

All children participated in an international multicenter study (Study on the Prevention of Allergy in Children in Europe (SPACE, Biomed II Program), dealing with the prevention of allergy by dust-mite avoidance measures.

Mothers were approached either at routine visits at two obstetric wards in Vienna (Vienna General Hospital, 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, skin prick testing or serum IgE measurement was performed. Families were included in case of a positive allergy history and positive allergy tests (*Dermatophagoides pteronyssinus*, *D. farinae*, birch, ryegrass, ragweed, and cat) in any parent. A first cross-sectional survey was carried out at a mean age of 20.6 days (SD: 7.1 days). Families were visited at home to implement allergy-prevention measures and perform nasal lavages. The main intervention measure studied was allergen avoidance by mattress covers (Allergy Control®, ALK), which were given in a randomized fashion to 50% of the participating families. Data on parental education, smoking habits, housing modalities, and pets in the home were gathered by a questionnaire. After 6 months, children were seen again at their homes, and a questionnaire regarding symptoms of the index child was filled out by the parents. Questions included the 6-month period prevalence of prolonged coughing (>14 days' duration) as observed by the parents, a history of wheezy breathing, shortness of breath, allergy to food, or a dry skin. A doctor's diagnosis of wheezy bronchitis and a diagnosis of atopic dermatitis were also asked for.

Informed consent was obtained from the parents prior to all measurements, and the study protocol was approved by the Vienna University's ethics committee and the ethics committee of the SMZ-Ost.

Nasal lavage

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. All allergen avoidance measures were introduced after performing the nasal lavage. Children were classified as being free of rhinitis, having serous rhinitis (watery secretions), or having purulent rhinitis (thick, yellowish secretions) on the basis of the inspection of their nasal lavage fluid. Nasal lavage was obtained by instilling with a needleless syringe 2 ml of 37°C physiologic saline solution into each nasal cavity with the baby lying supine. Wash fluid and secretions were immediately aspirated into a sterile specimen trap (tracheal suction set (Dahlhausen, Germany) and a 30-cm suction catheter (Uno, Germany) attached to a portable suction device (Atmos LC-16, Draeger). The samples were processed in a standardized way. The specimens were rested for at least 1 h at room temperature and were then taken back to the laboratory. Samples were weighed and diluted 1:1 with dithiothreitol (Cleland's Reagents, Biorath, Germany). They were centrifuged at 1454 *g* for 10 min at room temperature. Cells were discarded, and the supernatants were stored at -30° until analysis. Supernatant was 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.

Serum ECP and EPX concentrations were determined by a sensitive and specific double-antibody radioimmunoassay (Pharmacia Upjohn AB, Uppsala, Sweden), in which 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.

Statistics

Due to their skewed distribution, concentrations of ECP and EPX were compared between symptomatic and asymptomatic children by the Wilcoxon sign rank test. Analysis was performed with SAS software (version 6.0).

Results

Among the target population (average number of births/year: SMZ-Ost: 2000, Vienna General Hospital: 2000), screening questionnaires were distributed to a total of 4309 parents (1261 SMZ-Ost and 3048 Vienna General Hospital) between March 1997 and June 1998. Questionnaires were distributed in parallel in the obstetric wards and the children's wards of the two hospitals. Of these, 4159 were returned. A positive answer to any of the screening questions was given by 24.7% ($n=1227$). History of symptoms associated with allergic disease was confirmed by means of a positive skin prick test or specific IgE (RAST class ≥ 2) in 553 parents. A total of 408 parents consented to participate in the study. After birth, 11 children did not fulfill the inclusion criteria (due to low birth weight, admission to

the intensive care unit for more than 7 days). Hence, a total of 397 children entered the study. The average birth weight of the children was 3.37 kg (SD: 0.44 kg).

From the study population, 394 (99.2%) of children were seen again at the age of 6 months (range: 182–218 days). As recorded by questionnaire, a history of prolonged coughing was reported by 48.5% of parents. Wheezy breathing was observed by the parents in 17.7% and shortness of breath in 3.4% of all babies. A doctor's diagnosis of wheezy bronchitis was given in 10.8% and a diagnosis of atopic dermatitis in 3.2% of children, whereas a dry skin was noticed in 27.7%. In seven children, pneumonia had occurred during their first 6 months of life. Allergy to food was reported by seven parents.

A nasal lavage was performed in 375/394 children (95%). In the remaining 19 children, sufficient samples could not be obtained. There was no history of respiratory symptoms in any of the children at this point in time. At the home visit, 300 infants were free of any nasal discharge, 65 had some serous nasal secretions on inspection, and 10 had overt purulent rhinitis. The 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 rhinitis were not analyzed for the relationship between eosinophil markers and the occurrence of symptoms because of the likelihood of recent viral infection, their ECP values being about 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%) and showed a skewed distribution. The median was 0 μg/l and the 5–95th percentiles were 0–69.38 μg/l. The 75th percentile was 16.9 μg/l and the 90th percentile 46.08 μg/l. There was no correlation between ECP concentration and recovery ($r=0.06$; $P=0.24$). Factors associated with increased ECP or EPX were investigated by linear regression analysis. The results show that there were no effects of observer, smoking during pregnancy, birth weight, or size of household (as a proxy of social class) on ECP or EPX concentrations.

Children who were given a diagnosis of wheezy bronchitis during their first 6 months of life had increased nasal ECP values (Table 1, Fig. 1) at the age of 4 weeks. The prevalence of wheezy bronchitis was 8.3% in 192 children where ECP could not be detected,

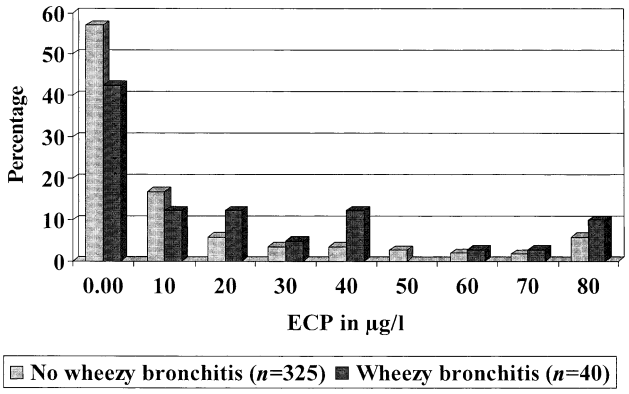

Figure 1. Distribution of nasal ECP in 365 children at 4 weeks of age and diagnosis of wheezy bronchitis at 6 months of age.

and 13.9% in 173 children where ECP was detectable. This difference was of borderline significance ($P<0.08$). The odds ratio for development of wheezy bronchitis if the ECP concentration was above the 75th percentile (16.9 μg/l) was 2.42 (95% CI: 1.25–4.7; $P=0.009$). Higher ECP values were also observed in children whose parents reported any cough during the first 6 months of life. No association was observed with development of atopic dermatitis or food allergy. However, patient numbers were rather small in these groups.

Very similar results were observed for EPX, the correlation coefficient with ECP being $r=0.81$ ($P<0.0001$). EPX was detected in 197 samples (54.7%), the median in these samples being 6.64 μg/l (5–95th percentiles: 0–73.2 μg/l). The odds ratio of EPX greater than the 75th percentile (16.48 μg/l) for developing wheezy bronchitis was 2.84 (CI: 1.38–5.84).

Discussion

In a selected sample of infants at high risk of developing allergies, we have observed that the level of eosinophil proteins on the nasal mucosa surface is associated with the development of wheezing at 6 months. This corroborates a recent finding of our group that serum ECP predicts persistent wheezing in children that were seen at their first wheezing episode (3). The upper airways, specifically the nasal epithelium, shares many properties

Table 1. Nasal EPX concentration (μg/l) at 4 weeks of age and allergic symptoms at 6 months of age

	Yes	EPX (median; 5–95th percentile)	No	EPX (median; 5–95th percentile)	*P* value Yes *vs* no
Prolonged coughing	176	8.76 (0–87.14)	189	6.72 (0–75.48)	<0.05
Any wheeze	64	8.12 (0–148.98)	301	7.74 (0–78.7)	NS
Diagnosis of wheezy bronchitis	40	12.14 (0–148.98)	325	7.5 (0–81.46)	<0.05
Shortness of breath	13	6.02 (0–57.4)	352	7.74 (0–87.04)	NS
Dry skin	102	8.32 (0–98.0)	263	7.13 (0–78.7)	NS
Atopic dermatitis	12	3.96 (0–98.0)	353	7.74 (0–78.7)	NS

with the lower airways in allergic disease; it is easily accessible and nasal lavage procedures are well tolerated even by small infants (7, 10, 11). Measurements of inflammatory markers on the upper airways respiratory epithelium can generally be assumed to reflect the process more directly. Therefore, the determination of inflammatory markers in the nasal epithelium fluid would be useful both for clinical and for epidemiologic purposes.

We can only speculate on the origin of nasal eosinophil proteins at the age of 4 weeks. T_h2 cytokines that are produced on the maternal–fetal interface during pregnancy presumably improve fetal survival by down-regulation of T_h1 cytokines (19). Thus, pregnancy is associated with a T_h2 phenomenon which may lead to a greater tendency for eosinophil activation after contact of the immature immune system with pathogens than in older children.

Nevertheless, in this prospective study, a higher level of eosinophil activation was observed in those who developed wheezy bronchitis. Significant postnatal exposure is rather unlikely since children were lavaged shortly after discharge from the obstetric wards. One possibility is prenatal exposure to allergens. Cord-blood studies by our own group have demonstrated that intrauterine priming of mononuclear cells by allergens may occur, and this might lead to early upregulation of proinflammatory cytokines (20). Alternatively, increased nasal ECP/EPX concentrations might reflect a genetic predisposition to allergies that is independent of allergen exposure.

Smoking during pregnancy is a known risk factor for recurrent wheezing in infancy, but this factor was not associated with nasal ECP/EPX and hence has not biased our findings. All nasal lavages were performed *before* the introduction of allergy preventive measures, which therefore could not have affected the results.

There is no standardized method for the collection of upper-airway epithelial lining fluid (6, 12–14). It may be collected either by nasopharyngeal aspiration or by instilling a predefined volume of either Ringer lactate or saline into the nasal cavity with either a needleless syringe or a pump spray. Fluid is then re-collected by a vacuum pump or by the microsuction method. Alternatively, when there is little secretion, the suction catheters may be rinsed with a specific volume of diluent after the collection of the specimen. Furthermore, secretions can be collected via absorption by cotton strips or foam-rubber samplers.

The main issue in the interpretation of lavage data is the unknown dilution when analyzing the samples. In a methodological paper by Heikkinen et al. (12) in children with an upper respiratory illness (URI), the dilution factor was 1.8–432 (median: 11.2). This elegant study suggested that adjustment of the marker of interest by total protein content of the sample produces the best correlation with the "true" concentration, as measured in direct nasopharyngeal aspirates in the same children at the same time points. However, their study can be applied only to children with URI. In our experience, total protein cannot always be detected in the NAL of healthy children. In our studies, adjustment for albumin did not increase the reliability of ECP concentrations. Others have suggested that inulin (10, 11) be added to the lavage fluid to adjust for dilution. Without correction for dilution, the intraindividual variability of any marker in the NAL will increase, thus decreasing the statistical power to detect differences between groups. If statistically significant differences between groups are observed, it is likely that a biologic effect is present. Dilution is also a factor in the measurement of low concentrations of proteins in the NAL, which very often will be below the detection limit of the specific assay. Klimek & Rasp (6) observed most values under the detection limit by the NAL technique. This is in keeping with our results, in which approximately 50% of the samples with ECP/EPX concentrations were below the detection limit.

Given these shortcomings, it is very surprising that markers in a single NAL specimen have been shown to distinguish between atopic and asthmatic children and demonstrate some association with the severity of disease (7, 8, 15, 16). Infants with bronchial obstruction during respiratory infection have significantly higher ECP in nasal washes than patients without obstruction (17). Reijonen et al. (15) measured nasopharyngeal ECP concentration in children with acute bronchiolitis, and found that a high nasal ECP level was a predictor of subsequent hospitalization and physician-diagnosed bronchial obstruction.

Whereas nasal lavage is an appropriate tool to study disease mechanisms of the upper airways, particularly those of allergic rhinitis (18), there are few data on the association between upper and lower airway inflammation in children. Exposure studies with ozone (9) or swine dust (5) have shown that changes in the nasal lavage reflect to some extent inflammation in the lower airways. In experimentally induced infection with rhinovirus, the virus RNA can be found in both the upper and lower airways (4). In wheezy infants, Balfour-Lynn et al. (11) have observed increased TNF-α concentrations in the NAL, particularly in children infected with the RS virus. These data suggest that local inflammation which promotes asthma exacerbation in the susceptible host can be studied by the nasal lavage technique.

In summary, we have shown that already at 4 weeks of age detectable concentrations of eosinophil proteins are present in almost every second child with increased risk of allergies. A value above the 75th percentile of ECP/EPX was associated with a twofold risk of developing a wheezing illness. Further follow-up with nasal lavages at 6, 12, 18, and 24 months will show whether the predictive value will change with increasing age of the children.

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