Original article

Serum eosinophil granule proteins predict asthma risk in allergic rhinitis

Background: Allergic rhinitis is a common disease, in which some patients will deteriorate or develop asthma. It is important to characterize these patients, thereby offering the possibility for prevention. This study evaluated eosinophil parameters as potential indicators of deteriorating allergic airway disease.

Methods: The subjects of the study included all patients who suffered seasonal allergic rhinitis and had participated in a study 6 years earlier, in which blood eosinophils, serum eosinophil cationic protein (ECP) serum eosinophil peroxidase (EPO), nasal lavage ECP and nasal lavage EPO levels were measured. Patients in the present study were interviewed on occurrence of rhinitis symptoms during the last season, rhinitis outside season, asthma-like symptoms and asthma diagnosis, and were skin-prick tested for common aeroallergens. Eosinophil parameters from the study 6 years earlier were then tested for the ability to predict occurrence of new allergies, worsening of rhinitis and occurrence of asthma.

Results: Forty-four patients participated in the study. In four patients seasonal rhinitis symptoms had deteriorated, 10 had experienced perennial rhinitis symptoms, 14 reported asthma-like symptoms and seven had been diagnosed with asthma. Thirteen had developed additional sensitization. Patients developing asthma-like symptoms compared with patients with no such symptoms had significantly higher serum ECP (16.7 µg/l vs 8.2 µg/l; $P \leq 0.01$) and serum EPO (17.9 µg/l vs 8.8 µg/l; $P \leq 0.05$). Results were similar, considering patients diagnosed with asthma. Blood eosinophils and nasal lavage parameters were not related to development of asthma and asthma-like symptoms. No eosinophil parameter was related to deterioration of rhinitis or additional sensitization.

Conclusion: Serum ECP and EPO in patients with seasonal rhinitis demonstrated a high predictive ability for later development of asthma.

L. P. Nielsen^{1,2}, C. G. B. Peterson³,
R. Dahl¹

Departments of ¹Respiratory Diseases and ²Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark; ³Department of Medical Sciences, Clinical Chemistry, Uppsala University, Uppsala, Sweden

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Lars Peter Nielsen
Dept. of Clinical Pharmacology
The Bartholin Building
Aarhus University
DK-8000 Aarhus
Denmark

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Allergic rhinitis is a common condition, affecting an increasing part of the population in industrialized countries (1–3). In the vast majority of patients, symptoms ease off and eventually disappear over the years. However, in a subset of patients, symptoms may deteriorate or seasonal symptoms may become perennial or both. Likewise, the presence of allergic rhinitis is a strong risk indicator for later onset of asthma (4–6).

At present, monitoring allergic rhinitis and assessment of disease activity are mainly based on subjective parameters, i.e. symptoms. However, studies have demonstrated eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) in both serum and nasal lavage fluid as sensitive indicators of the disease activity in allergic rhinitis (7, 8). Inflammation is a main feature of allergic rhinitis and asthma and eosinophils constitute a hallmark of this inflammation. Therefore, parameters characterizing eosinophil activity, e.g. ECP and EPO, could prove

valuable as prognostic indicators, especially, when considering the risk of deterioration of allergic rhinitis or onset of asthma. This would delimit a population of allergic rhinitis patients, with an increased risk, who might benefit from intensified intervention. This study intended to estimate eosinophil parameters in blood and nasal lavage fluid as risk indicators for deterioration of allergic rhinitis severity, development of additional allergies or onset of asthma over a 6-year period.

Materials and methods

Patients

All 44 patients participating in this study had participated in a study 6 years earlier (8). Briefly, all patients at that time suffered from seasonal allergic rhinitis for at least 2 years, were monoallergic to grass pollen (*Phleum pratense*) and had not received corticosteroids

for at least 2 months. In the previous study, blood eosinophils (b-EOS), serum eosinophil cationic protein (s-ECP), serum eosinophil peroxidase (s-EPO), nasal lavage ECP (n-ECP) and nasal lavage EPO (n-EPO) were measured 2 weeks prior to the pollen season.

Design

This study included one visit at which patients were interviewed for their actual disease activity, and a skin-prick test including pollens (grass, birch, mugwort), animal dander (cat, dog, horse), house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*) and moulds (*Cladosporium herbarum*, *Alternaria alternata*) was performed. The skin-prick test was identical to the one performed in the study 6 years ago. The visit took place 2 weeks prior to the pollen season, i.e. at the same time of the year as the preseasonal visit of the previous study.

Methods

The interview included questions about allergic rhinitis symptoms during season and their severity during the latest season, when compared to those 6 years ago. Moreover, patients were asked whether they had experienced allergic rhinitis symptoms off-season and whether these were perennial. Patients were also asked whether they had experienced asthma-like symptoms, defined as episodes of wheezing, cough or dyspnoea within the recent 6 years and whether they had been diagnosed with asthma by a physician during the period. New allergies were confirmed by a positive skin-prick test or radioallergosorbent test (RAST) towards the allergen. Results on b-EOS, s-ECP, s-EPO, n-ECP and n-EPO were derived from the previous study.

The number of eosinophil granulocytes in peripheral blood (EOS) was determined by counting in a Fuchs-Rosenthal chamber. Briefly, peripheral blood was diluted (1:18) in a hypotonic solution containing acetone as stabilizer and eosin for staining of eosinophils. Between 20 min and 2 h later, the number of eosinophils was counted using a Fuchs-Rosenthal chamber with a volume of 3.2 μ l. All counts were performed in duplicate.

For measurements of s-ECP and s-EPO, serum was obtained after allowing venous blood to clot for 60 min at 20°C, followed by centrifugation (10 min, 4°C, 1600 g). The resulting serum samples were kept at -80°C until analysis. Nasal lavage fluids were centrifuged (10 min, 4°C, 1600 g). After centrifugation, the supernatant was kept at -80°C until analysis for ECP and EPO. ECP was measured by using the Pharmacia CAP System® ECP FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), according to the instructions of the manufacturer. The inter- and intra-assay coefficients of variation were less than 8% and the detection limit was 0.5 μ g/l. EPO was assessed by prototype immunofluorometric assays utilizing the Pharmacia CAP System®. Briefly, purified EPO was used as standard, ranging from 0.5 to 200 μ g/l.

Monoclonal antibody (mAb) was produced by the standard hybridoma technique by the fusion of spleen cells from EPO immunized Balb/c mice with Sp2/0 mouse myeloma cells, followed by cloning. Supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for activity against purified EPO. Selected hybridomas were expanded and antibodies purified from these were of the IgG1 subtype.

The assay procedure was identical to that of Pharmacia CAP System ECP FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). One mAb was covalently bound to the ImmunoCAP® (Pharmacia & Upjohn Diagnostics AB), and the other mAb was labelled with the enzyme 6-galactosidase. Briefly, specific antibodies

coupled to the solid phase react with EPO in standard or sample, and bound protein is detected by means of the enzyme-labelled mAb followed by the addition of the development solution. The fluorescent product is eluted with Stop solution and measured in the FluoroCount96 (Pharmacia & Upjohn Diagnostics AB). The fluorescence detected is then directly proportional to the amount of EPO in the standard and sample. Crossreactivities with ECP and myeloperoxidase (MPO) in the EPO assay were $\leq 0.3\%$ and $\leq 0.01\%$, respectively. The detection limit of the EPO assay was 0.5 μ g/l, and the intra- and interassay coefficients of variation were less than 10%. Five serum samples were diluted serially two, four and eight times in phosphate buffer, pH 7.5, containing 0.2% cetyltrimethylammonium bromide (CTAB), 10 mM Na-ethylenediaminetetraacetic acid (EDTA), 20% glycerol and 5% foetal calf serum (FCS) to test for parallelism, with the standard curve as an indication of specificity. The mean ratio between obtained and expected levels was 1.02 (range 88–112%). The level of each marker was assessed for predictive value upon occurrence of new allergies, change in rhinitis symptom severity and occurrence of asthma.

Statistical analysis

Data were analysed by Student's *t*-test or, if more than two groups, by analysis of variance. Data were log-transformed, if an assumption of normal distribution was not met. If log-transformation of data did not lead to the achievement of normal distribution, non-parametric statistics were applied (Wilcoxon rank sum test). Analyses were carried out using SPSS software (version 10.0, SPSS Inc., Chicago, IL, USA).

Ethics

The study was approved by the local scientific ethics committee (County of Aarhus). All patients gave informed consent in writing before entering the study.

Results

Diagnosis and symptoms

All participants of the previous study were contacted; however, 19 patients were not willing to participate in the follow-up study for unknown reasons. Forty-four patients participated in this 6-year follow-up study (Table 1). All patients still suffered from allergic rhinitis during the grass pollen season.

Only four patients had experienced a deterioration in grass pollen-induced rhinitis, whereas 18 had felt an improvement in the condition and the condition of 22 others remained unchanged. Rhinitis symptoms outside the grass pollen season had developed in 17 patients during the past 6 years and 10 of these patients experienced perennial symptoms. Considering asthma, 14 patients reported the first appearance of episodes of cough, wheezing and/or dyspnoea during the past 6 years and seven of these had been diagnosed as having bronchial asthma by a physician. Nineteen patients had never experienced asthma or asthma-like symptoms, while the remaining 11 patients reported the presence of asthma or asthma-like symptoms earlier than the past 6 years.

Table 1. Characteristics of the study population

<i>n</i> (male/female)	44 (25/19)
Age, years [mean (range)]	41.7 (25–64)
Race	
Caucasian	43
African-American	1
Smoking (yes/no)	6/38
Asthma*	
Start of symptoms	14
Diagnosis	7
Serum IgE (kU/l)	91.2 (10.4)
RAST grass (kU/l)	9.1 (1.1)
Blood eosinophils ($\times 10^9/l$)	0.14 (0.01)
Serum ECP ($\mu g/l$)	14.30 (2.13)
Serum EPO ($\mu g/l$)	15.08 (2.21)

Results are given as mean (SEM), unless otherwise stated.

*Start of symptoms or diagnosis within the past 6 years.

No correlations were observed among patients experiencing increasing severity of rhinitis symptoms, patients with rhinitis outside season and patients reporting asthma or asthma-like symptoms (data not shown). New sensitization, defined by positive skin-prick test, was observed in 13 patients.

Eosinophil markers

Patients who developed asthma-like symptoms for the first time during the follow-up period, had almost twice as high a level of ECP and EPO in serum compared with those with no asthma-like symptoms (Fig. 1). Results were similar, when considering the subset of patients diagnosed with asthma during the follow-up (Fig. 1). In addition, all patients with s-ECP $> 20.7 \mu g/l$ ($n = 7$) or s-EPO $> 18.7 \mu g/l$ ($n = 7$) developed asthma symptoms.

Figure 1. Serum-ECP, serum-EPO and blood eosinophils in the previous study according to development of asthma symptoms or asthma diagnosed by a doctor 6 years later. ■ Asthma reported ($n = 14$); ■ asthma diagnosed ($n = 7$); □ no asthma ($n = 19$); mean (SEM). * $P < 0.05$; ** $P < 0.01$.

S-ECP and s-EPO were not related to rhinitis symptoms during the pollen season (Table 2). Patients with deteriorating rhinitis symptoms as well as those developing rhinitis symptoms outside the grass pollen season tended to have higher levels of ECP and EPO in nasal lavage fluids (Table 3). However, these differences did not reach a statistical significance. Likewise, patients who were sensitized to allergens other than grass pollen during the follow-up period tended to differ in n-ECP and n-EPO levels (Table 3).

Blood eosinophils did not differ between patients grouped by any disease parameter. Considering patients who refused to participate in the follow-up study, there were no significant differences either in blood eosinophils or in eosinophil markers in serum and nasal lavage fluid, when compared with participants (data not shown).

Table 2. Blood and serum eosinophil parameters 6 years ago compared with changes in disease manifestations during the past 6 years

	<i>n</i>	Blood eosinophils ($10^9/l$)	Serum ECP ($\mu g/l$)	Serum EPO ($\mu g/l$)
Rhinitis symptoms during the grass pollen season				
Deteriorating	4	0.12 (0.04)	9.77 (3.71)	7.73 (3.21)
Unchanged	22	0.13 (0.02)	9.39 (3.60)	9.43 (1.93)
Improving	18	0.15 (0.02)	12.04 (3.72)	15.00 (2.12)
Rhinitis symptoms outside grass pollen season				
Yes	17	0.13 (0.02)	10.00 (3.61)	10.50 (2.21)
No	27	0.14 (0.02)	10.29 (3.75)	11.35 (2.18)
Additional sensitization (skin-prick test)				
Yes	13	0.12 (0.02)	11.52 (3.31)	11.16 (1.53)
No	20	0.14 (0.02)	10.38 (3.51)	11.36 (2.05)

Values are given as mean (SEM).

Table 3. Nasal lavage eosinophil parameters 6 years ago compared with changes in disease manifestations during the past 6 years

	<i>n</i>	Nasal ECP ($\mu g/l$)	Nasal EPO ($\mu g/l$)
Rhinitis symptoms during grass pollen season			
Deteriorating	4	4.0 (20.5)	1.8 (33.0)
Unchanged	22	4.0 (2.7)	1.0 (2.3)
Improving	18	4.0 (2.6)	1.0 (2.3)
Rhinitis symptoms outside grass pollen season			
Yes	17	5.1 (8.8)	2.1 (9.0)
No	27	4.0 (1.5)	1.0 (0.8)
Asthma			
Start of symptoms	14	4.6 (3.5)	1.4 (8.3)
Diagnosis	7	5.0 (6.0)	1.0 (4.5)
Never asthma	19	4.0 (1.4)	1.0 (1.9)
Additional sensitization (skin-prick test)			
Yes	13	5.0 (10.9)	2.4 (4.0)
No	20	4.0 (1.9)	1.0 (0.7)

Values are median (interquartile range).

Discussion

This is the first study to demonstrate the ability of eosinophil markers, i.e. s-ECP and s-EPO, to predict the risk for future asthma in patients with allergic rhinitis. This is consistent with previous data that demonstrated s-ECP as a predictor for the development of persistent wheezing in infants suffering viral bronchiolitis (9). Likewise, s-ECP has been able to distinguish infants developing asthma following acute bronchial obstruction from those who did not (10). The blood eosinophils count did not demonstrate this predictive ability. This is probably because of the fact that the serum levels of ECP and EPO reflect not only the number of eosinophils, but more importantly their state of activation also (11). In addition, serum levels of ECP have been shown to correlate with the number of activated eosinophils present in the bronchial mucosa of asthmatics (12). Moreover, our findings seem to be substantiated by the consistent presence of both ECP and EPO in serum and in nasal lavage.

The reason why levels of ECP and EPO in serum but not in nasal lavage fluid were predictive of the development of asthma remains more speculative. A possible explanation could be that, whereas nasal lavage fluid levels represent the local inflammatory 'burden', serum levels reflect the systemic nature of inflammation, including the presence of subclinical inflammation in bronchial mucosa. Several studies have demonstrated an increased bronchial hyper-responsiveness in non-asthmatic patients with allergic rhinitis as well as a correlation between the level of hyper-responsiveness and sputum eosinophils (13–15). Activated eosinophils in bronchial tissue have been observed in patients with clinical asthma as well as in atopic non-asthmatics, demonstrating the presence of subclinical mucosal inflammation (16), and it would seem natural to expect this in future patients with symptomatic asthma. Furthermore, patients with allergic rhinitis showed the same level of increase in broncho-alveolar lavage (BAL) eosinophils as allergic asthmatics following bronchial segmental allergen challenge (17).

Taken together, the observations favour the 'United Airways' theory, stating allergic rhinitis and asthma as expressions of one underlying disease (18). Expectedly, blood eosinophils as well as their granule proteins would reflect this. However, whereas ECP and EPO in serum reflect both state of activation and number of eosinophils, blood eosinophils only represent the latter.

The presence of more severe inflammation of the nasal mucosa in patients developing asthma seems an unlikely explanation for our findings, as nasal lavage fluid levels of ECP and EPO had no predictive value for any of the parameters considered. Likewise, sampling was performed out of season in asymptomatic patients to avoid an influence of differences on seasonal exposure. The presence of higher sensitivity of eosinophils in individuals developing asthma leading to an enhanced risk of allergic

inflammation in other organs could seem yet another explanation for our findings.

A very high proportion of patients (14/44), who never had asthma or asthma-like symptoms prior to the study 6 years earlier, experienced asthma during the 6-year follow-up. Similar results have been demonstrated in studies with longer follow-up periods (19–21). It could be speculated that patients refusing to participate in the follow-up study were those with the mildest degree of disease, not experiencing additional problems, i.e. perennial rhinitis or asthma, and thereby leading to the findings of a higher incidence of asthma and extra-seasonal rhinitis. However, this seems to be somewhat contradicted by the consistent levels of eosinophils and their markers, when comparing participants and non-participants.

Considering the natural history of allergic rhinitis, most studies demonstrate an improvement of rhinitis symptoms in a majority of patients (19, 22, 23). This was also seen in our results, as more than 90% of patients reported seasonal symptoms to be either unchanged or improved within the recent 6 years, albeit all patients still experienced symptoms during season. A considerable number of patients had experienced rhinitis symptoms off-season and 22.7% developed perennial rhinitis. This is in accordance with results from a previous study in children with a somewhat longer observational period (21), although considerably higher than the incidence seen in a study with a similar follow-up period (24).

In conclusion, patients in this study with seasonal allergic rhinitis and monosensitization to grass pollen demonstrated a high risk for the development of additional sensitizations, perennial rhinitis and bronchial asthma. These changes in disease manifestations occurred within a relatively short time span of 6 years.

Serum markers of systemic eosinophil inflammation, i.e. s-ECP and s-EPO, measured out of season at the beginning of the 6-year period, demonstrated a high predictive ability for development of bronchial asthma during follow-up. Such patients should be identified and offered preventative measures. One possibility would be to investigate the preventive abilities of different interventions in a large-scale, double-blind, placebo-controlled, confirmative study.

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Conflict of interest

At the time of the study, Dr C.G.B. Peterson was an employee of Pharmacia&Upjohn AB.

References

1. Lundback B. Epidemiology of rhinitis and asthma. *Clin Exp Allergy* 1998;**28**(Suppl 2):S3–S10.
2. Skoner DP. Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis. *J Allergy Clin Immunol* 2001;**108**(Suppl 1): S2–S8.
3. Linneberg A, Nielsen NH, Madsen F, Frolund L, Dirksen A, Jorgensen T. Increasing prevalence of allergic rhinitis symptoms in an adult Danish population. *Allergy* 1999;**54**: 1194–1198.
4. Plaschke PP, Janson C, Norrman E, Bjornsson E, Ellbjar S, Jarvholm B. Onset and remission of allergic rhinitis and asthma and the relationship with atopic sensitization and smoking. *Am J Respir Crit Care Med* 2000;**162**:920–924.
5. Settipane RJ, Hagy GW, Settipane GA. Long-term risk factors for developing asthma and allergic rhinitis: a 23-year follow-up study of college students. *Allergy Proc* 1994;**15**:21–25.
6. Guerra S, Sherrill DL, Martinez FD, Barbee RA. Rhinitis as an independent risk factor for adult-onset asthma. *J Allergy Clin Immunol* 2002;**109**:419–425.
7. Nielsen LP, Bjerke T, Christensen MB, Pedersen B, Rasmussen TR, Dahl R. Assessment of the allergic reaction in seasonal rhinitis: acoustic rhinometry is a sensitive and objective method. *Clin Exp Allergy* 1996;**26**:1268–1275.
8. Nielsen LP, Bjerke T, Christensen MB, Skamling M, Peterson CG, Mygind N et al. Eosinophil markers in seasonal allergic rhinitis. Intranasal fluticasone propionate inhibits local and systemic increases during the pollen season. *Allergy* 1998;**53**:778–785.
9. Pifferi M, Ragazzo V, Caramella D, Baldini G. Eosinophil cationic protein in infants with respiratory syncytial virus bronchiolitis: predictive value for subsequent development of persistent wheezing. *Pediatr Pulmonol* 2001;**31**: 419–424.
10. Pohunek P, Kucera P, Sukova B, Votava F, Zikan J. Serum ECP taken in the acute episode of bronchial obstruction can predict the development of bronchial asthma in young children. *Allergy Asthma Proc* 2001;**22**:75–79.
11. Venge P, Bystrom J, Carlson M, Hakansson L, Karawacjzyk M, Peterson C et al. Eosinophil cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. *Clin Exp Allergy* 1999;**29**:1172–1186.
12. Hoshino M, Nakamura Y. Relationship between activated eosinophils of the bronchial mucosa and serum eosinophil cationic protein in atopic asthma. *Int Arch Allergy Immunol* 1997;**112**:59–64.
13. Hara J, Fujimura M, Myou S, Furusho S, Abo M, Oribe Y et al. Eosinophilic inflammation, remodeling of lower airway, bronchial responsiveness and cough reflex sensitivity in non-asthmatic subjects with nasal allergy. *Int Arch Allergy Immunol* 2006;**140**:327–333.
14. Polosa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C et al. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000;**15**:30–35.
15. Tatar M, Petriskova J, Zucha J, Pecova R, Hutka Z, Raffajova J et al. Induced sputum eosinophils, bronchial reactivity, and cough sensitivity in subjects with allergic rhinitis. *J Physiol Pharmacol* 2005;**56**(Suppl 4):227–236.
16. Djukanovic R, Lai CK, Wilson JW, Britten KM, Wilson SJ, Roche WR et al. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur Respir J* 1992;**5**:538–544.
17. Becky Kelly EA, Busse WW, Jarjour NN. A comparison of the airway response to segmental antigen bronchoprovocation in atopic asthma and allergic rhinitis. *J Allergy Clin Immunol* 2003;**111**:79–86.
18. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;**108**(Suppl 5):S147–S334.
19. Danielsson J, Jessen M. The natural course of allergic rhinitis during 12 years of follow-up. *Allergy* 1997;**52**:331–334.
20. Greisner WA III, Settipane RJ, Settipane GA. Co-existence of asthma and allergic rhinitis: a 23-year follow-up study of college students. *Allergy Asthma Proc* 1998;**19**:185–188.
21. Linna O, Kokkonen J, Lukin M. A 10-year prognosis for childhood allergic rhinitis. *Acta Paediatr* 1992;**81**:100–102.
22. Greisner WA III, Settipane RJ, Settipane GA. Natural history of hay fever: a 23-year follow-up of college students. *Allergy Asthma Proc* 1998;**19**:271–275.
23. Simola M, Holopainen E, Malmberg H. Changes in skin and nasal sensitivity to allergens and the course of rhinitis; a long-term follow-up study. *Ann Allergy Asthma Immunol* 1999;**82**: 152–156.
24. Hagy GW, Settipane GA. Risk factors for developing asthma and allergic rhinitis. A 7-year follow-up study of college students. *J Allergy Clin Immunol* 1976;**58**:330–336.