

Twenty-four-hour time course of eosinophil granule proteins ECP and EPX during bronchial allergen challenges in serum of asthmatic children

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To study *in vivo* monitoring variables for bronchial allergen challenges, we investigated the time course of the eosinophil granule proteins, eosinophil cationic protein (ECP) and eosinophil protein X (EPX) after allergen provocation in serum. Thirty-two asthmatic children sensitive to house-dust mites and six healthy young adult controls were challenged by bronchial allergen provocations with *Dermatophagoides pteronyssinus* and *D. farinae*. Blood samples were taken at regular intervals up to 24 h. Base-line concentrations of ECP ($P < 0.004$), EPX ($P < 0.002$), and eosinophils ($P < 0.001$) were found to be increased in asthmatic children, as compared with healthy controls. ECP and EPX concentrations showed a uniform pattern with two characteristic features: 1) a rapid increase for both mediators up to 30 min after provocation over base-line values ($P < 0.0001$ and $P < 0.001$), followed by a rapid decrease nearly to base-line values in the next 30 min; and 2) a steady increase for ECP and EPX up to 10 h ($P < 0.02$ and $P < 0.01$), and even higher levels at 24 h, after challenge ($P < 0.002$ and $P < 0.003$). We conclude that although eosinophils are activated in asthmatic children after bronchial allergen challenge, ECP and EPX concentrations are not suitable monitoring variables. Base-line eosinophils seem to predict the occurrence of a late-phase asthmatic reaction after allergen provocation.

Key words: allergen; asthma; challenge; children; eosinophils; eosinophil cationic protein (ECP); eosinophil protein X (EPX); house-dust mite; provocation.

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Mucosal inflammation caused by mediator release is considered to be an important feature of bronchial asthma. Several studies have provided evidence for the major role of the eosinophil granulocyte in inflammatory processes observed in asthma (3, 18, 28). Clinical findings could be confirmed by histologic investigations, which showed a predominant eosinophilia in the lungs of asthmatic patients (16). The eosinophil granulocyte contains several highly cytotoxic proteins such as eosinophil cationic protein (ECP), eosinophil protein X (EPX), eosinophil peroxidase (EPO), and major basic protein (MBP) (29). There is evidence that secretion of eosinophil-derived proteins (e.g., ECP, MBP) leads to damage of the respiratory epithelium (15, 26), which sustains and aggravates bronchial hyperreactivity in chronic asthma.

In bronchoalveolar lavage (BAL) of adult asthmatic patients during the late asthmatic reaction

(LAR) after allergen provocation, some authors found an eosinophilia (10) as well as elevated concentrations of eosinophil-derived mediators (12). Peripheral blood eosinophils correlate with nonspecific bronchial hyperresponsiveness in asthmatic subjects (23). Since occurrence of LAR after allergen exposure seems to be one of the determinants for developing chronic asthma, the aim of our study was to look for an *in vivo* variable for monitoring bronchial allergen challenges. Therefore we investigated the time course of serum ECP and EPX concentrations as well as eosinophil blood cell counts up to 24 h after allergen challenge.

Material and methods

Patients and control subjects

Thirty-two children (26 boys, 6 girls) aged 9–17 years (mean 12 years) were selected for the study. All the

children suffered from mild to moderate bronchial asthma, as defined by the American Thoracic Society (1). Base-line FEV₁ ranged from 65 to 120% predicted (mean 93%). House-dust mite sensitivity was the predominant sensitivity of these children and was verified by detection of specific IgE antibodies. CAP classes for *Dermatophagoides pteronyssinus* (*Dpt*) and *D. farinae* (*Df*) ranged from 2 to 6 (mean 4.5). Domestic house-dust mite exposure to *Der p* I ranged from 0 to 29 µg/g dust (mean 3.0 µg/g dust), and to *Der f* I from 0 to 42 µg/g dust (mean 6.9 µg/g dust) on the mattresses of the children.

Six healthy adults (two men, four women) aged 24–43 (mean 28) served as controls. For ethical reasons (allergen provocation, bloodletting, inpatient treatment), we did not choose healthy children, but fell back on young adult volunteers. All subjects were nonatopic (total IgE < 100 kU/l) and were non-smokers. No specific IgE antibodies were found against *Dpt* or *Df* (CAP class 0). Base-line FEV₁ ranged from 85 to 118% predicted (mean 95%). Data of patients and controls are summarized in Table 1.

Table 1. Clinical data of patients and controls

Pat.	Age (yr)	Sex (M/F)	Total IgE (kU/l)	Specific IgE (CAP class) <i>Dpt/Df</i>	FEV ₁ (% pred.) base line	PC ₂₀ FEV ₁ (SQU)
F.A.	10	M	1048	4/4	94	1000
A.A.	9	F	218	4/4	99	1000
O.A.	10	M	431	4/3	104	> 100 000
O.A.	10	M	634	6/5	115	1000
A.B.	14	F	819	5/4	104	8000
A.B.	12	M	1323	6/5	92	1000
T.B.	17	M	806	6/6	85	500
N.B.	12	F	921	5/6	67	1000
J.C.	13	M	120	3/2	102	20 000
G.D.	14	M	352	4/4	103	> 100 000
T.D.	16	F	1871	5/6	120	10 000
M.E.	13	M	405	5/4	71	> 100 000
F.F.	10	M	331	4/4	95	> 100 000
D.G.	13	M	—	—/4	86	10 000
O.H.	10	M	417	4/4	102	20 000
F.H.	12	M	956	6/5	68	> 100 000
B.I.	11	M	816	5/5	82	600
H.J.	9	M	226	4/4	81	50 000
S.K.	11	M	976	6/6	92	30
T.L.	10	M	185	4/4	83	> 100 000
J.M.	11	M	913	5/4	71	> 100 000
H.M.	11	F	19	3/2	96	4000
M.N.	10	F	349	6/3	85	200
R.P.	10	M	782	5/5	76	2000
J.S.	9	M	392	5/4	75	30 000
D.S.	9	M	601	5/4	99	40 000
D.S.	10	M	189	5/4	90	60 000
D.S.	17	M	1325	5/4	65	60 000
H.T.	9	M	1214	6/4	105	> 100 000
A.Y.	12	M	1801	6/5	92	200
S.B.	9	M	253	5/4	77	> 100 000
S.K.	12	M	976	5/6	116	100
T.L.	10	M	207	4/4	80	300
M.C.	11	M	1947	5/5	95	300
F.H.	12	M	956	6/5	99	2000
B.I.	11	M	816	5/5	74	500
D.S.	10	M	224	4/4	90	700
G.D.	14	M	308	4/4	100	1000
S.N.	26	F	19	0/0	117	> 100 000
R.H.	43	F	40	0/0	117	> 100 000
I.K.	24	F	16	0/0	82	> 100 000
N.G.	40	M	22	0/0	97	> 100 000
K.R.	21	F	20	0/0	118	> 100 000
A.L.	28	M	22	0/0	117	> 100 000

Study design

Thirty-two asthmatic children and six healthy controls were challenged with *Dpt* allergen extract, and six of the patients were also challenged separately with *Df* allergen. All challenges started at 0900. Patients were followed for 24 h. During the procedure, peak flow was measured at hourly intervals up to 12 h, as well as at 24 h, after provocation. Blood samples were drawn at regular intervals during the observation period.

Methods

Lung function tests were performed in a body plethysmograph (E. Jaeger, Würzburg, Germany). After base-line lung function (inhalation of the diluent), a titrated provocation was undertaken with a jet nebulizer (Pari-boy, Medanz, Starnberg, Germany). Each concentration was inhaled for 5 min. PC_{20} FEV₁ determined the early asthmatic reaction (EAR), while a 24-h peak-flow protocol detected a possible LAR.

Biologically standardized extracts of *Dpt* or *Df* (ALK, Copenhagen, Denmark) in concentrations ranging from 10 to 100 000 SQU were used. Because most patients showed specific IgE antibodies against both *Dpt* and *Df* in corresponding CAP classes, *Dpt* extract was chosen for provocation. Six patients were also challenged separately with *Df*, according to their predominant domestic exposure to *Df*.

Blood samples (3 ml) were drawn from an antecubital venous catheter at 14 time points (−60, 0, 10, 20, 30, 60, 120, 180, 240, 360, 480, 600, 720, and 1440 min). Serum was prepared according to guidelines recently published (19), and it was stored at −20°C until analyzed.

Eosinophil mediators (ECP, EPX) were determined from serum by radioimmunoassay (Pharmacia, Uppsala, Sweden) (19, 30). All assays were run in duplicate. The coefficient of intraassay variation was 7.9% for ECP and 4.3% for EPX; the interassay coefficient of variation was 4.7% for ECP and 6.8% for EPX. Eosinophil counts from differential blood cell count were determined at base line, at PC_{20} FEV₁, 3 h later, and at 24 h after provocation.

For statistical analysis, Wilcoxon's signed rank test, the Mann-Whitney U test, the linear rank correlation test, and multiple regression analysis were performed on a personal computer with the statistical package SPSS.

Results

Base-line serum concentrations of ECP ($P < 0.004$), EPX ($P < 0.002$), and eosinophil blood cell count (EOS) ($P < 0.001$) were found to be significantly

higher in the 38 asthmatic children than in the six healthy controls (Fig. 1). Base-line FEV₁ did not correlate to EOS, ECP, or EPX in asthmatic children. Comparing base-line EOS and ECP/EPX, we found no significant correlation either.

As a result of bronchial allergen challenge, the asthmatic children as a group demonstrated a strong EAR and LAR, whereas lung function in the controls did not vary significantly (Fig. 2). We measured ECP and EPX concentration at 14 time points up to 24 h. Because of lack of further information and as a means to make figures easier to read, five representative time points are depicted. Serum ECP ($P < 0.0001$) and EPX ($P < 0.001$) showed a significant early peak within 30 min in 27 patients, while in two patients the early peak occurred between 60 and 180 min. Nine children did not show an early peak. This early peak was followed by a rapid decrease to base-line values within the next 30 min (Fig. 2).

Thereafter a continuous increase of both mediators was noted (600 min: ECP $P < 0.02$ and EPX $P < 0.01$; at 24 h: ECP $P < 0.002$ and EPX $P < 0.003$) (Fig. 2). ECP and EPX concentrations of control subjects remained within the normal range and showed no significant variation over the 24-h observation period.

For all subjects, we found an extensive parallel time course of ECP and EPX serum concentrations from base line up to 24 h after provocation, with EPX being on a higher level.

The eosinophil blood cell count decreased slightly, but not significantly, in asthmatic children after challenge, with a minimum after 3 h ($P = 0.07$) (Fig. 2). At 24 h, there had been a significant increase of eosinophils over base-line values ($P < 0.0002$). In healthy controls, there were no apparent variations.

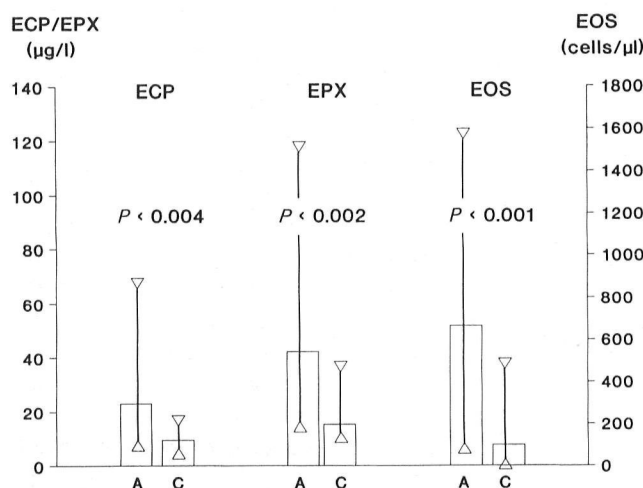


Fig. 1. Base-line serum concentrations of ECP, EPX, and blood eosinophil counts in 38 asthmatic children (A) and six healthy controls (C) (median, range).

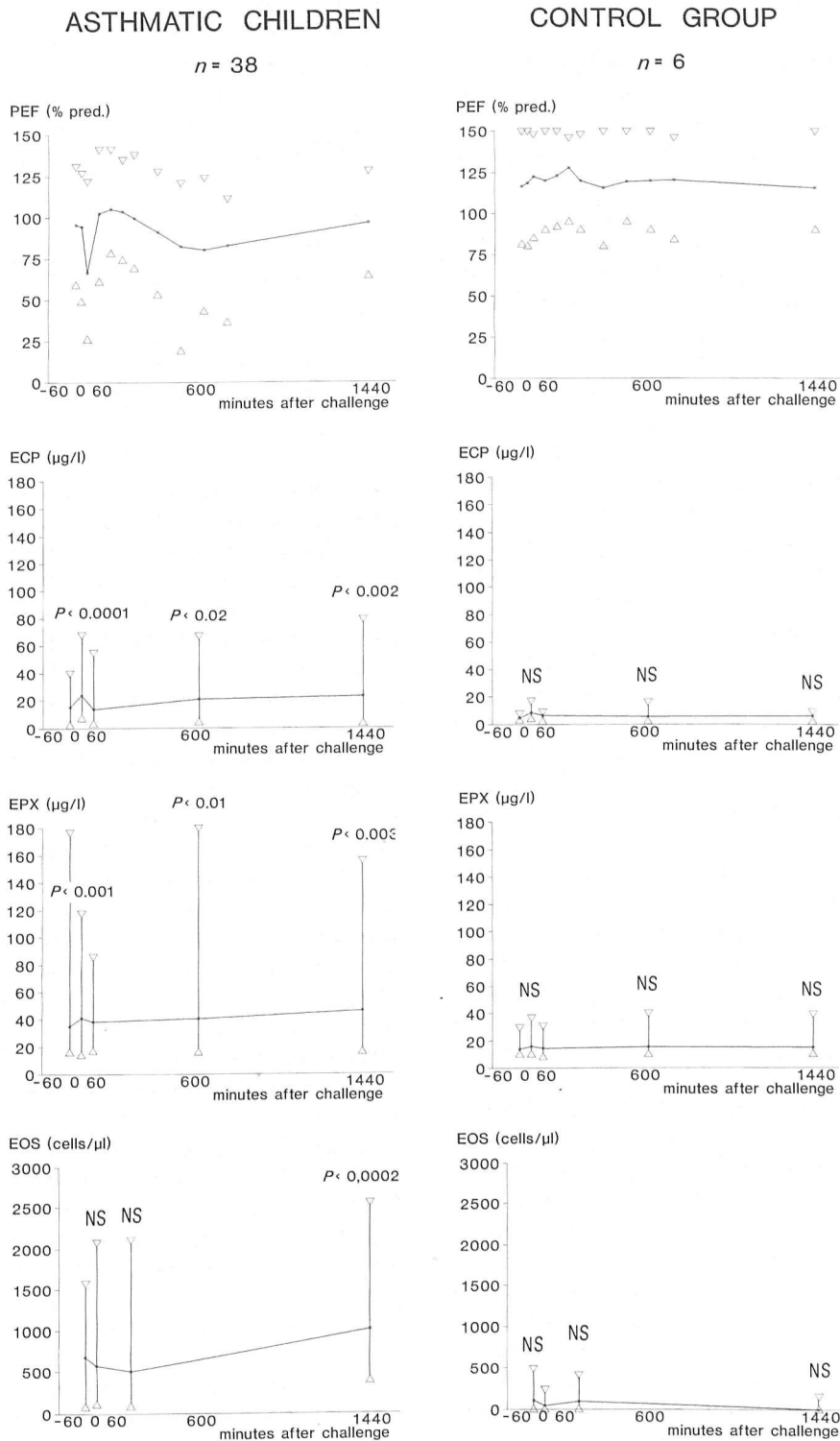


Fig. 2. Variation of ECP, EPX, blood eosinophil counts, and PEF in asthmatic children ($n = 38$) and healthy controls ($n = 6$), as compared with base-line values (median, range).

For study of the relationship between type of reaction and time course of mediator release, the group of asthmatic children was subdivided according to lung function pattern: six children developed an EAR, six children an LAR, and 23 children a dual

asthmatic reaction (DAR), while three asthmatic children sensitive to house-dust mite did not show any bronchoconstrictor response to allergen provocation.

In all asthmatic children exhibiting any broncho-

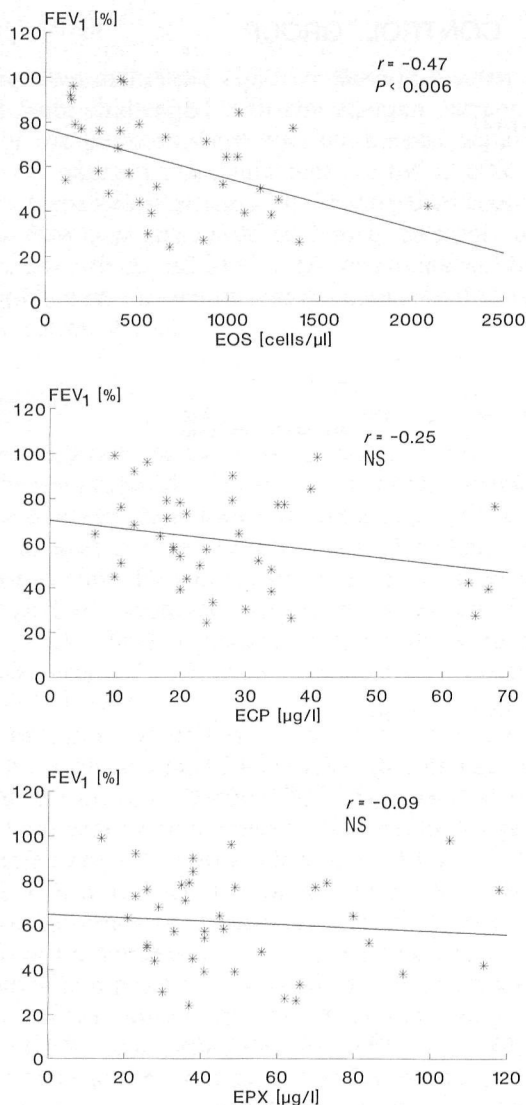


Fig. 3. Inverse correlation of FEV_1 to EOS ($n = 34$), ECP ($n = 38$), and EPX ($n = 38$) at 30 min after challenge (EAR) in asthmatic children.

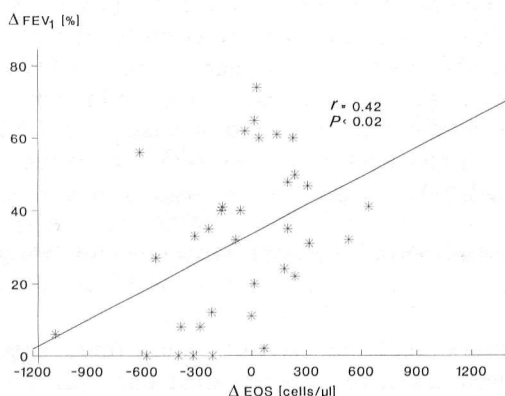


Fig. 4. Correlation of changes in EOS (EAR minus base-line values) and FEV_1 (decrease from base line to EAR) in asthmatic children ($n = 34$).

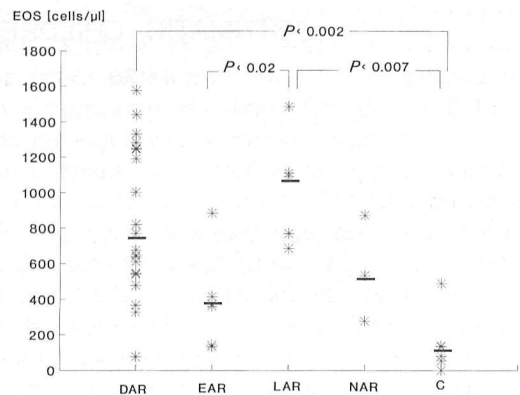


Fig. 5. EOS at base line according to clinical type of reaction (median).

constrictor response to allergen challenge (EAR, LAR, DAR), we could demonstrate a pattern similar to that for the group of children as a whole, while the three children with no asthmatic reaction (NAR) showed no significant variation in eosinophils and mediator release, like healthy controls.

Comparing lung function (FEV_1) and EOS during the first 30 min after provocation (EAR), we found a significant inverse correlation ($r = -0.47$ and $P < 0.006$), but not for ECP and EPX (Fig. 3). For the individual dynamic variation in terms of differences between EOS (from base line to EAR) and FEV_1 (from base line to EAR), there was also a significant correlation ($r = 0.42$ and $P < 0.02$) (Fig. 4). This correlation was again absent for ECP and EPX. Subjects with a strong increase of EOS from base line to EAR showed a strong fall in FEV_1 , and patients with a decrease of EOS showed only a slight decrease of FEV_1 .

Base-line EOS was significantly higher in asthmatic children exhibiting LAR ($P < 0.007$) or dual asthmatic reaction (DAR) ($P < 0.002$) than in healthy controls (Fig. 5), while asthmatic children without LAR (EAR, NAR) did not show significantly elevated EOS at base line. LAR and EAR were also statistically different in base-line EOS ($P < 0.02$) (Fig. 5). ECP and EPX were not suitable for forecasting LAR.

Multiple regression analysis revealed that ECP and EPX gave no additional information in distinguishing asthmatic children who were prone to develop an LAR after allergen challenge, as compared with EOS alone.

We could not find any correlation between specific IgE and eosinophil numbers or eosinophil granule proteins at any time point.

Discussion

The data of this study confirm that base-line eosinophils as well as serum concentrations of ECP and

EPX are significantly elevated in asthmatic children, as compared with healthy controls (Fig. 1). These results agree with published data from asthmatic adults (8, 17) and asthmatic children (22). To our knowledge, there are no comparable data in the literature concerning EPX serum concentration in asthmatic children.

In the search for a potential monitoring variable, we investigated the time course of ECP, EPX, and EOS in peripheral blood during and up to 24 h after bronchial allergen challenge. Irrespective of type of clinical reaction, we found a nearly uniform pattern of ECP and EPX serum levels, a pattern which is very similar to data published by Dahl et al. (9).

The rapid increase of both mediators during the first 30 min after challenge (Fig. 2) is supposed to be the result of degranulation of EOS in the lung. Carlson et al. showed that EOS of asthmatic patients have an increased propensity to release their granule proteins, which the authors suggest could be the consequence of priming of these cells (8). Further support comes from studies of bronchial biopsies in asthmatic patients, which demonstrated a positive proof of activated (EG2-positive) eosinophils (5). The rapid increase of ECP and EPX was followed by a rapid return to values near the base line. This can be related to eliminating processes such as those known for ECP (20).

While there are some reports about an increase of EOS and eosinophil-derived mediators during LAR (6–12 h) in BAL (10, 11), there are few data regarding serum concentration of eosinophil granule proteins in asthmatic children, especially not for a time course of 24 h after bronchial allergen challenge. We found an absolute increase of ECP and EPX serum levels at 600 min and an even more pronounced rise at 24 h after provocation, while in the literature a relative increase of eosinophil-derived mediators after an early decrease was demonstrated (13). We again assume that this steady increase of serum ECP and EPX concentrations is due to degranulation of EOS, which are recruited into lung tissue for days after bronchial allergen challenge, as is known from BAL studies (6, 10, 11, 21).

Unexpectedly, there was a small subgroup of three asthmatic children sensitive to house-dust mites who did not show a clinical response to bronchial allergen challenge (NAR). In terms of EOS and eosinophil-derived mediators, they showed exactly the same pattern as the healthy controls. Despite the small number of patients, these data could indicate a link between the relatively low serum concentration of ECP and EPX in asthmatic children and the absence of a significant asthmatic reaction.

Comparing both serum eosinophil-derived mediators (ECP and EPX), we found an approximate parallel course of serum concentration at all time

points over the 24-h observation period, emphasizing the common cell origin. EPX concentrations were significantly higher than ECP. These findings agree with other studies (8, 17, 25).

EOS decreased slightly, but not significantly, in asthmatic children after allergen challenge, with a minimum after 3 h. This can be interpreted as a beginning influx into lung tissue, as was proposed before from BAL studies after allergen challenge (6, 10, 11, 21). At 24 h, we observed a significant increase of peripheral blood EOS in asthmatic children over base-line values, as has been observed before for asthmatic adults (12, 13, 14). Whether this increase was due to an enhanced release from bone marrow, as was suggested from studies in guinea pigs (2), is not clear.

To find a suitable monitoring variable during allergen challenges, we investigated the relationship between lung function and EOS as well as ECP/EPX-serum concentrations. A significant correlation was found only for EOS and FEV₁ during EAR, but not for ECP/EPX or for EOS at other time points (e.g., LAR).

The literature reveals contradictory results regarding the relationship between clinical indicators and serum or BAL concentrations of eosinophil granule proteins. A significant correlation was found for ECP in BAL and FEV₁ as well as the Aas-asthma score in a study in asthmatic adults from France (4). Two other studies showed a significant correlation between decreased serum ECP concentration and reduction of asthmatic attacks, and improvement in FEV₁ after treatment with inhaled steroids (22, 27).

Our negative results, however, agree with data from Carlson et al., who could not find a significant correlation between lung function and secretory activity of eosinophils in asthmatic adults (7, 8). These contradictory results could be due to problems in measuring serum ECP or EPX concentrations, which may derive from the well-known, high intraindividual coefficient of variation and from the suspected presence of unknown binding proteins in plasma.

We were not able to demonstrate different patterns of mediator release according to clinical type of reaction after allergen provocation. Only EOS were significantly increased in those asthmatic children who developed an LAR after allergen challenge (Fig. 5). This latter finding was shown before by Venge & Dahl (24).

In conclusion, our data indicate that measuring serum ECP and EPX concentrations during and after bronchial allergen challenge is not a useful method in monitoring individual bronchial allergen provocation. Prechallenge peripheral blood EOS seem to be the more appropriate variable to distinguish asthmatic children who are prone to develop LAR. Further detailed kinetic studies of peripheral

blood eosinophils and their granule proteins up to 48 h after allergen challenge are needed to assess the value of monitoring time course after bronchial allergen provocation.

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