

Eosinophil activity markers in peripheral blood have high predictive value for bronchial hyperreactivity in patients with suspected mild asthma

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The present study aimed to evaluate the predictive value of eosinophils and markers of their activity for bronchial hyperreactivity (BHR) in a population of patients with recently developed clinical symptoms of asthma. The activation of eosinophils was estimated by measuring eosinophil cationic protein (ECP) in serum. In addition, flow cytometry was used to measure the expression of the EG2-epitope on intracellular ECP in eosinophils from peripheral blood. Twenty-eight consecutive patients with clinical history of asthma were studied. Of the 28 patients, 18 had a positive bronchial challenge test measured as $PD_{20} \leq 1600 \mu\text{g}$ histamine. A significantly higher concentration of eosinophils and a trend to higher ECP in the peripheral blood was found in the hyperreactive group than in the nonreactive group. However, the intracellular expression of ECP did not correlate with the PD_{20} value, and no significant difference between the groups was found. With one eosinophil activity marker, either serum ECP or EG2, BHR could be predicted in 70% of the patients. If we combined any two of the activity markers (serum ECP, EG2, or the percentage of eosinophils), the predictive value increased to 100%. We conclude that the blood eosinophil concentration, as well as, to some extent, serum ECP, has a high specificity for BHR in patients with recently developed clinical symptoms of asthma. Despite normal bronchial reactivity, some patients had signs of activated eosinophils, i.e., high serum ECP and increased EG2 expression. Thus, these markers may reflect early stages in the development of BHR. Our results also indicate that a combined evaluation of percentage of eosinophils and of eosinophil activity markers is of clinical value to predict BHR.

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Allergic and nonallergic asthma are characterized by increased airway responsiveness to various specific and nonspecific stimuli. This increase usually accompanies asthma symptoms but is not synonymous with asthma (1). The precise mechanism underlying the hyperresponsiveness is still unclear, but it is believed that inflammation of the airways plays a major role. There has recently been an emphasis on the importance of inflammation in asthma and the contribution that the eosinophils make to this. Blood and tissue eosinophilia is often found in asthma (2–4), and the number of eosinophils in peripheral blood correlates with the

severity of asthma (2, 4, 5). Furthermore, activated eosinophils have been demonstrated in increased numbers (5–7). The granules of the eosinophil granulocyte contain several basic proteins which are biologically active products, such as eosinophil cationic protein (ECP), which has become widely accepted as an indicator of eosinophil activity (8). The monoclonal antibody EG2 (anti-ECP/EPX) is believed to detect activated eosinophils (9), and increased intracellular binding of EG2 to peripheral blood eosinophils from asthmatic patients, in comparison with healthy blood donors, has been reported (10–12). Moreover, several studies have

shown a correlation between the number of eosinophils in the blood, their state of activation, and the severity of the asthmatic condition (4, 13, 14) and also between the eosinophil count in asthmatic patients and their responsiveness to inhaled histamine and methacholine (8, 15).

The present study investigated whether eosinophil activity markers could be used to predict the risk of developing bronchial hyperreactivity (BHR) in patients with asthma-like symptoms. We also wanted to establish whether we could find in a clinical material a correlation between BHR, evaluated by bronchial histamine challenge, and eosinophil number and activation in peripheral blood, as measured by detection by the FOG method (10, 16) of increased serum ECP levels and the expression of intracellular ECP and EG2 antibodies.

Material and methods

Patients

Twenty-eight consecutive steroid-naïve patients with symptoms suggestive of asthma, referred to the allergy clinic at Karolinska Hospital for investigation and help in diagnosis, were included in the study. There were 20 women and eight men with a mean age of 31.4 (range 18–47) years. They all had a clinical history of asthma-like symptoms, such as a variable feeling of tightness in the chest, wheezing, breathlessness, and cough when exposed to various stimuli. In some cases, the patients did not have actual symptoms at the time of investigation. All patients were prick tested with a standard panel of eight airborne allergens (Soluprick, ALK, Denmark), and atopy was confirmed, with at least one positive response, in 19 cases. Seven of the patients were smokers. The patients had a mean prechallenge FEV₁ of 93% (range 67–113%) of predicted value. Patients with a history of infection within 2 weeks before the study were not included. None of the patients were taking steroids, theophyllines, or chromones. β_2 -Agonists were withheld for at least 12 h before the bronchial challenge, and oral antihistamines for at least 2 weeks.

The study was approved by the local ethics committee, and the patients gave informed consent.

Bronchial provocation tests

For determination of nonspecific bronchial responsiveness, increasing doses of histamine were inhaled to obtain the PD₂₀ value (the provocative dose of histamine resulting in a 20% reduction in FEV₁). A dosimeter, a jet nebulizer Spira Elektro 2 (Respiratory Care Center, Hameenlinna,

Finland), was used for the inhalation test. At a driving pressure of 200 kPa, this jet nebulizer gives an airflow of 7.5 l/min and generates an aerosol with 95% of the particles smaller than 2 μ m. The aerosol delivery duration and the start of the nebulization are adjustable, and the inspiratory flow and the inspired volume are also controlled. This permits a controlled mode of inhalation and an accurate calculation of the dose inspired (17). After recording of the baseline FEV₁, the subjects inhaled saline, followed by increasing doses of histamine for a specified number of breaths from an initial dose of 11 μ g to a maximum of 990 μ g. FEV₁ was measured 2 min after each dose of histamine. The test was interrupted when FEV₁ fell by at least 20% of the prechallenge value or when a maximum cumulative histamine dose of 2000 μ g was reached. The logarithmic histamine doses were plotted against the percentage of postsaline FEV₁, and PD₂₀ values were calculated from the cumulative doses by linear interpolation. The patients with PD₂₀ FEV₁ $\leq$ 1600 μ g histamine were considered hyperreactive.

Bronchial reactivity was measured between 9:00 and 11:30 a.m. In pollen-allergic patients, measurements were taken outside the pollen season.

Eosinophil counts

Blood samples for differential counts were drawn just before the histamine challenge. The eosinophil percentage and number were obtained by routine differential counting with a Coulter STKS (five-part differential count) at the Department of Clinical Chemistry, Karolinska Hospital.

Serum ECP

ECP levels were measured with a commercially available fluoroimmunoassay (Pharmacia ECP CAP System FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden). The sera were collected and handled according to the manufacturer's instructions and stored at -20°C until analyzed.

Intracellular expression of the EG2 epitope on ECP in peripheral blood eosinophils

The method used for fixation and cell membrane permeabilization of peripheral blood leukocytes (the FOG method) has been described in detail elsewhere (10, 11). Briefly, permeability and separated eosinophils were obtained by treating paraformaldehyde-fixed blood leukocytes with *n*-octyl-beta-D-glucopyranoside (OG). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody EG2 (Pharmacia Diagnostics AB,

Table 1. All individual data

Patient	Allergy	PD ₂₀	% eos	Tot. eos	s-ECP	EG2
		μg		10 ⁹ /l	μg/l	MFI units
1	Yes	2000	2.5	0.1	3.0	41.1
2	—	2000	2.6	0.1	7.3	23.4
3	Yes	2000	1.8	0.2	23.0	25.0
4	Yes	2000	1.5	0.1	4.7	28.5
5	—	2000	3.8	0.1	10.0	44.4
6	No	2000	2.2	0.1	6.2	34.1
7	Yes	2000	3.0	0.3	8.8	36.2
8	—	2000	4.3	—	8.5	34.5
9	No	2000	1.8	0.1	8.1	25.4
10	Yes	2000	3.8	0.3	—	28.2
11	Yes	860	2.6	0.1	9.6	34.3
12	Yes	800	3.8	0.1	6.4	35.1
13	Yes	76	11.4	0.4	24.0	36.6
14	No	1400	0.4	—	4.4	19.8
15	Yes	100	4.5	0.3	12.0	29.7
16	Yes	1200	9.5	0.5	28.0	26.2
17	Yes	90	1.2	0.1	7.1	17.5
18	No	710	5.1	0.4	14.0	29.3
19	Yes	600	9.4	0.5	6.2	35.5
20	Yes	800	1.2	—	7.4	22.3
21	Yes	780	7.4	0.2	12.0	38.6
22	Yes	1400	8.0	0.5	18.0	33.3
23	Yes	460	12.8	1.4	30.0	43.0
24	Yes	1100	6.4	0.3	5.5	55.2
25	Yes	1600	7.5	0.6	18.0	31.5
26	Yes	110	6.2	0.3	50.0	37.0
27	No	200	13.3	1.0	66.0	35.6
28	No	115	3.4	0.2	15.0	45.1

eos: eosinophils; Tot. eos: total number of eosinophils.

Uppsala, Sweden) was added to the permeabilized leukocytes, and the eosinophils were analyzed by flow cytometry for the expression of antibody bound to intracellular ECP. The final leukocyte preparations were analyzed in an Epics XL (Coulter Inc., Hialeah, FL, USA) flow cytometer. A minimum of 500 eosinophils were analyzed, and the expression of intracellular EG2-epitope on ECP was measured as mean fluorescence intensity (MFI) units.

Statistics

Results are presented as median values and inter-quartile ranges (i.q.r.) unless otherwise stated. Statistical differences were calculated with the non-parametric Mann–Whitney test. Correlations between the different parameters were sought by calculating the Spearman’s rank correlation coefficient. Values of *P* < 0.05 were considered significant.

Results

All the individual data are summarized in Table 1 and in Figs. 1–3.

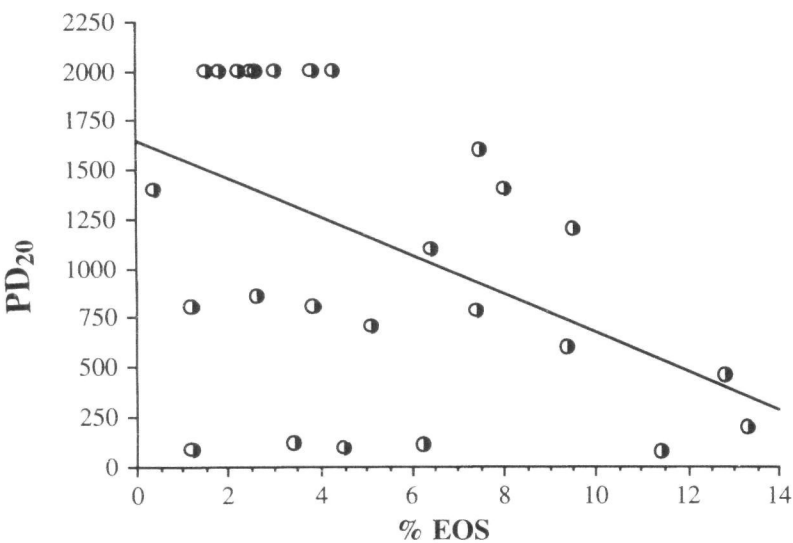


Fig. 1. Correlation between PD₂₀ and percentage of eosinophils (*r* = −0.4, *P* < 0.05).

Bronchial provocation tests

Despite their clinical history, only 18 of the 28 patients had a positive bronchial histamine challenge test result, measured as PD₂₀ ≤ 1600 μg histamine. The median histamine PD₂₀ value was 1150 μg (i.q.r. 530–2000 μg).

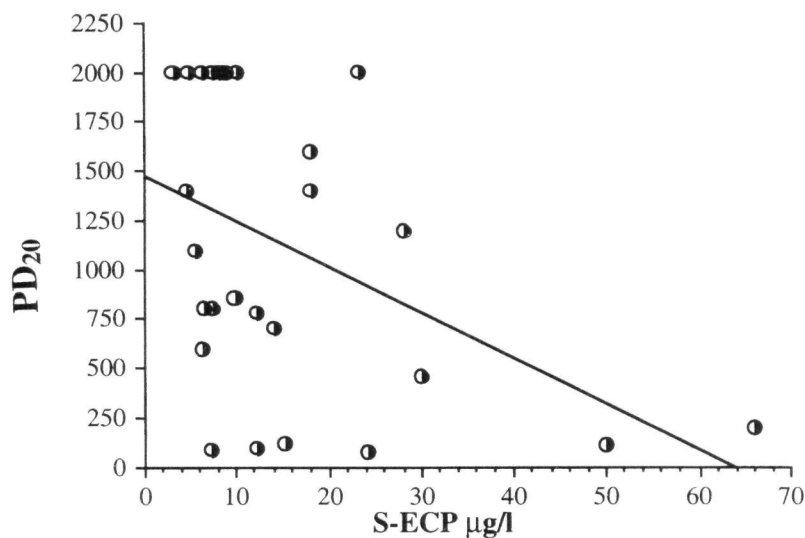


Fig. 2. Correlation between PD₂₀ and serum ECP ($r = -0.4$, $P = 0.051$).

Eosinophil counts

The proportion of eosinophils in the peripheral blood was 3.8% (i.q.r. 2.4–7.5), and a correlation was found between PD₂₀ and the proportion of eosinophils ($P < 0.05$) (Fig. 1). No significant difference was detected between the normoreactive and the hyperreactive group (Fig. 3a). The concentration of eosinophils ($0.3 \times 10^9/l$, i.q.r. 0.1 – $0.43 \times 10^9/l$) was significantly higher ($P < 0.01$) in the peripheral blood from the hyperreactive than in that from the normoreactive group (Fig. 3b).

Serum ECP

The median value for serum ECP was $9.6 \mu g/l$ (i.q.r. 6.6 – $18.0 \mu g/l$). There was a correlation that almost reached significance between PD₂₀ and S-ECP ($P = 0.051$) (Fig. 2). A nonsignificant difference between the hyperreactive and the normoreactive group was found ($P = 0.07$) (Fig. 3c). S-ECP correlated significantly with the total number of eosinophils ($P = 0.001$).

Intracellular expression of the EG2 epitope on ECP

The mean fluorescence intensity units (MFI) value for EG2 was 34.2 (i.q.r. 27.2 – 36.8) units. There was no significant correlation between PD₂₀ and EG2 ($P = 0.51$). No significant difference was found between the groups (Fig. 3b).

Predictive value of eosinophil activity markers for BHR

In this study, 10/18 patients in the histamine-reactive group had an increased proportion of eosinophils in peripheral blood compared to the reference value for healthy subjects at our hospital

Table 2. Predictive value of eosinophil activity markers

	No. of patients with one positive marker	No. of patients without positive marker
Nonreactive group PD ₂₀ >1600 μg histamine	5	5
Reactive group PD ₂₀ $\leq 1600 \mu g$ histamine	12	6
	No. of patients with at least two positive markers	No. of patients with fewer than two positive markers
Nonreactive group PD ₂₀ >1600 μg histamine	0	10
Reactive group PD ₂₀ $\leq 1600 \mu g$ histamine	10	8

With one positive eosinophil activity maker, either serum ECP or EG2, to predict BHR, predictive value (PV) was 70%. $PV = 12/(5 + 12) = 70$. With two positive eosinophil markers, serum ECP, EG2, or percentage of eosinophils to predict BHR, predictive value was 100%. $PV = 10/(0 + 10) = 100$.

(1–6%) (Fig. 3a), and 6/16 had a higher concentration of eosinophils (0.05 – $0.40 \times 10^9/l$) (Fig. 3b). In the nonreactive group, the concentration and percentage of eosinophils were within the normal ranges.

Increased serum ECP levels were detected in 7/18 patients in the reactive group and in 1/9 patients in the nonreactive group, in comparison with healthy controls (2 – $16 \mu g/l$).

The intracellular binding of EG2 to eosinophils was increased in 9/18 patients in the reactive group and in 4/10 patients in the nonreactive group (Fig. 3c). The normal range given for intracellular binding of EG2, 27.3 ± 7.0 MFI units (mean $\pm$ SD), is based on results from 18 healthy blood donors analyzed during the time period of this study (Fig. 3d).

If either serum ECP or EG2 was used as one marker, 70% of the patients with BHR were predicted. If any two of the eosinophil activity markers (serum ECP, EG2, or eosinophil percentage) were combined, the predictive value increased to 100% (Table 2).

Discussion

The eosinophil granulocyte is considered to be of central importance in the development of bronchial asthma. In symptomatic atopic patients, there is an increased local accumulation of eosinophils in the submucosa of the bronchi (3). These cells are probably activated, and some of their secreted products, ECP and major basic protein (MBP), can be measured in increased concentrations in broncho-alveolar and nasal fluid (18–22).

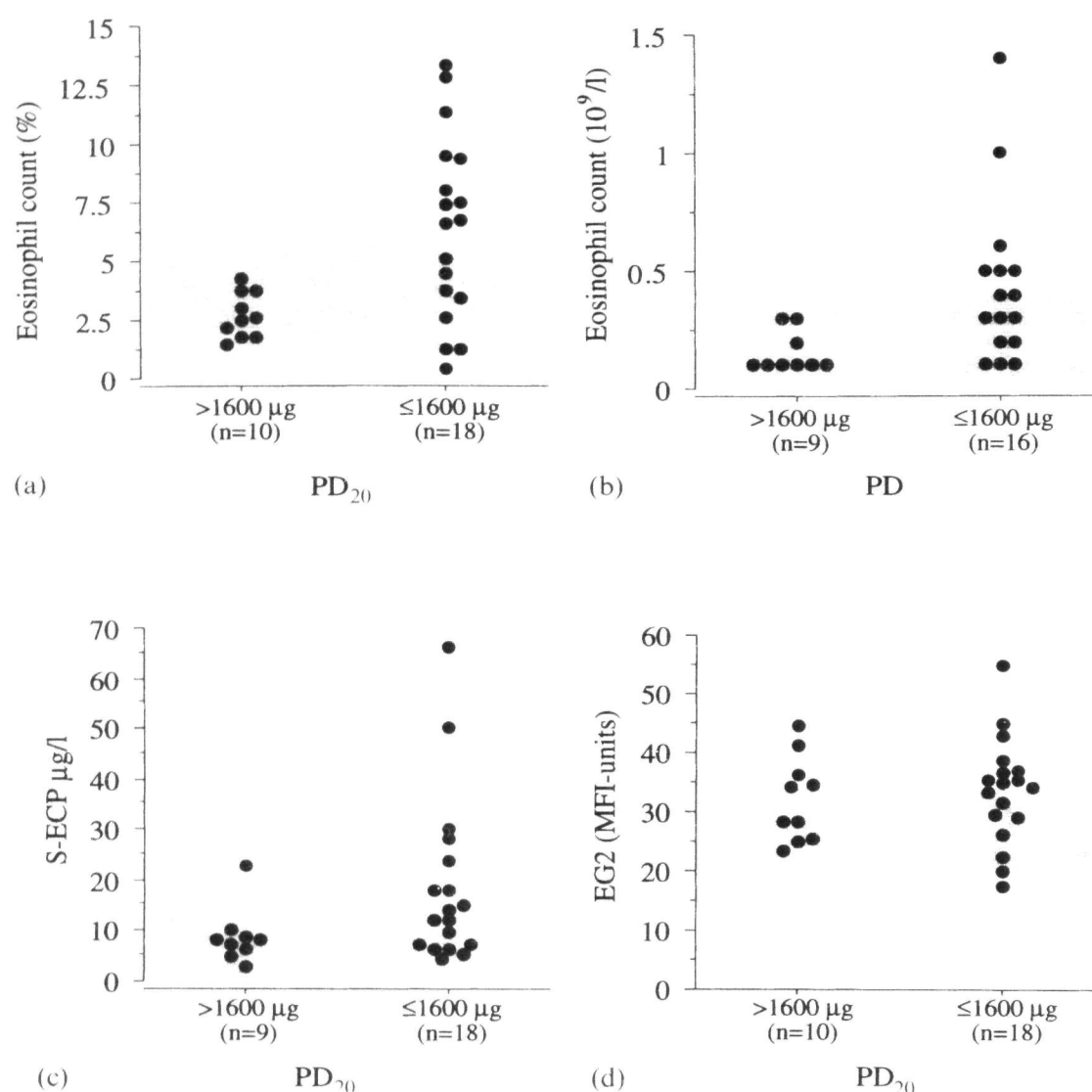


Fig. 3. Individual values. Shaded areas: normal ranges. Statistical differences between normoreactive and hyperreactive groups were calculated with nonparametric Mann-Whitney test. a) Percentage of eosinophils (NS). b) Concentration of eosinophils ($P < 0.01$). c) Serum ECP (NS). d) EG2 (MFI units) (NS).

The asthma diagnosis is based on a combination of various symptoms and signs. There is still not one single test that allows us to diagnose or classify the severity of asthma, and not even the presence or absence of BHR can be regarded as a standard criterion for the diagnosis. Therefore, our aim was to investigate whether eosinophil activity markers in peripheral blood could be used as biologic markers of BHR in patients with asthma-like symptoms.

If we used the conventional way to correlate the two parameters as in most studies, we found, like many others (2–4, 9, 13), a correlation between eosinophilia and hyperreactivity. However, the correlation between ECP in serum and BHR was weak and only close to significance ($P = 0.051$). This fits well with previous findings of a strong correlation between ECP and bronchial reactivity (6, 8, 15), although other studies have not found this (6, 23). Thus, the ECP level depends on both the total number of eosinophils and their state of activation. In contrast to earlier allergen provocation studies (7, 23), we failed to demonstrate in our patients a correlation

between BHR and eosinophil activity, measured as increased intracellular expression of the EG2 epitope on ECP. The intracellular expression of the EG2 epitope on ECP in eosinophils in peripheral blood is probably an earlier and possibly a more sensitive marker of activated eosinophils than serum ECP. This was previously shown by Ihre et al. (23) in asthmatic patients exposed to low doses of allergen and in patients during the pollen season (7). A study by Skedinger et al. (12) investigated patients with acute severe asthma with exacerbation needing steroid treatment, whereas our patients were stable without anti-inflammatory treatment. Skedinger et al. found values above normal for EG2 in patients before treatment, but these decreased significantly after a prednisolone course.

The most probable cause of the lack of correlation found in the present study is the difference in patient material from earlier studies. In Sweden, most patients who develop asthmatic symptoms and signs of BHR inhale steroids early in the disease process. Because of the entry criteria chosen in this study – patients had to be steroid,

chromone, and theophylline naive – most subjects had mild BHR, and some could be considered not to have asthma or evident BHR despite their clinical symptoms. Our patients were thus at one end of the disease spectrum, a fact which could lead to a weak correlation between parameters regarded as reflecting disease. Another important factor could be the intensity of the stimulus, which was probably low in our patient group. Furthermore, different inflammatory stimuli might affect the various stages of activation differently.

However, since one aim of the study was to evaluate the diagnostic value of eosinophil markers in patients with asthma-like symptoms, we also analyzed data with respect to positive predictive values for BHR. In this way, we found that with the use of one marker of primed eosinophils, such as EG2 or serum ECP, the positive predictive value for BHR in these patients was 70%. If we combined any two of the eosinophil activity markers (serum ECP, EG2, or the percentage of eosinophils), the positive predictive value increased to 100%. These findings emphasize the importance of eosinophil activation in the development of asthma and BHR. Using a combination of eosinophil markers to predict BHR is consequently of clinical value in patients with newly developed, mild, asthma-like symptoms.

Nevertheless, increased MFI values for EG2-positive cells did not discriminate between patients with or without hyperreactivity, indicating that this marker is a very early sign in the disease development. The number of eosinophils clearly discriminates between hyperreactive and nonreactive patients, indicating a closer relation to the asthma diagnosis. The diagnostic value of serum ECP in this population seems to be somewhere between the clinical relevance of EG2 and eosinophil concentration.

Our results indicate the importance of the different biologic roles of various eosinophils markers in the diagnosis and clinical activity of asthma. In this patient group with relatively weak symptoms, measurement of eosinophil concentration in blood, as well as, to some extent, serum ECP, was a more useful test than the more sophisticated measurements of intracellular ECP in active form. However, a combination of eosinophil markers can be used to predict BHR in patients with newly developed, asthma-like symptoms.

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