Eosinophil degranulation as an allergy activation marker

Admiraal, C.J.

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Chapter 7

Surplus value of eosinophil blood count and serum ECP level to diagnose and monitor asthmatic patients

C.J. Pronk-Admiraal¹, Tj. Haitjema², P. Horikx†² and P.C.M. Bartels¹

Departments of Clinical Chemistry¹ and Respiratory Medicine²
Medical Centre Alkmaar, The Netherlands
† deceased

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Summary

Background
Eosinophil Cationic Protein is one of the granule proteins in eosinophilic granulocytes. Release of this protein during blood clotting may reflect the activity state of eosinophilic granulocytes in diseased individuals. The purpose of this study was to evaluate the additional value of serum ECP over blood eosinophil count, (specific) IgE concentration and CRP concentration in order to follow the effects of corticosteroid treatment in asthmatic patients and to distinguish individual patients with asthma from healthy subjects on the basis of laboratory results.

Methods
In a longitudinal study, serial measurements of serum Eosinophil Cationic Protein concentration, blood eosinophil count and other laboratory parameters have been evaluated and compared with spirometry and tests of bronchial hyperresponsiveness in 10 asthmatic subjects. The patients were investigated before therapy was started and 3, 6 and 9 months after the start of therapy with inhaled corticosteroids. Laboratory parameters of the patient group are compared with results obtained from a reference group of apparently healthy adults (n = 223).

Results
Statistically significant correlations were observed between blood eosinophil counts and serum Eosinophil Cationic Protein concentrations with the hyperresponsiveness tests PC20 (r = 0.44 and r = 0.46, respectively) and with a decrease in FEV1 after exercise (r = 0.66 and r = 0.60, respectively). A significant difference was detected between serum ECP concentrations from the patient group and from the reference group. However, a wide range of overlapping results was observed between the reference group and the asthmatic patients.
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Conclusions
Asthma is a disease that is now more frequently treated by general practitioners. In general practice, one has not always the opportunity to evaluate asthma activity by application of hyperreactivity tests. When hyperreactivity testing is not available, measuring serum Eosinophil Cationic Protein concentration or eosinophil blood count would be an alternative method to monitor effects of corticosteroid treatment. In this study the additional value of serum ECP in addition to blood eosinophil count was evaluated to determine the effects of treatment in patients who started with application of corticosteroids. However, because analogous correlation coefficients of laboratory parameters with tests regarding hyperresponsiveness were found, no additional value of serum Eosinophil Cationic Protein concentration over eosinophil blood count in monitoring the effect of corticosteroids was detected. The additional value of serum ECP concentrations and eosinophil blood counts to detect an asthmatic constitution for individual cases is doubtful.
Introduction

Asthma is a disease characterized by a variable degree of severity of airway obstruction, airway inflammation and bronchial hyperresponsiveness (1, 2). The precise mechanism underlying the hyperresponsiveness is still unclear, but it is believed that inflammation of the airways plays a major role (3, 4).

The presence of infiltrating eosinophils in biopsies of bronchial mucosa and eosinophilia in blood and sputum of asthmatic patients reflects the involvement of these cells in the asthma inflammatory reaction, especially in late-phase reactions (5, 6, 7).

The blood eosinophil count is considered to be a simple technique to monitor asthma activity (5, 8, 9, 10). Eosinophil pathogenicity is caused by the release of its granular content (11). The granules of eosinophilic granulocytes contain several biologically active basic proteins, such as Eosinophil Cationic Protein (ECP) (12). The quantification of this protein in serum is accepted as an indicator of eosinophil degranulation activity (13). A blood test that could assess the activity of disease in asthmatic patients would be of great diagnostic value. ECP concentration can be established in serum (14). It has been suggested that serum ECP concentration is an additional tool for assessing the activity of bronchial asthma (15, 16, 17). However, there are also studies in which serum ECP appears to be a poor indicator of disease activity for asthmatic patients (18). We hypothesize that it might be valuable to establish the ECP release per eosinophil for determination of disease activity grade.

Corticosteroid inhalation therapy has improved the quality of life for many asthmatic patients by reducing the severity of airway inflammation. Treatment with corticosteroids does not only reduce the clinical symptoms but also decreases serum ECP levels (19). We performed a longitudinal study in asthmatic patients with serial measurements of serum ECP, eosinophil count and other laboratory parameters in comparison with estimation of lung function and bronchial hyperreactivity to assess the effect of corticosteroid treatment. Serum CRP concentrations were measured as a marker of
inflammation and (specific) IgE concentrations may indicate the atopic component in allergic asthma. The purpose of this study was to evaluate the additional value of serum ECP levels over eosinophil blood counts, (specific) IgE concentrations and CRP concentrations to evaluate the effect of treatment in patients starting to use corticosteroids and to distinguish individual patients with asthma from healthy subjects on the basis of laboratory results. We also studied whether laboratory parameters, such as eosinophil blood count or ECP serum concentration, are good alternatives to diagnose or to monitor asthma activity instead of lung function tests.

Materials and Methods

Subjects
Adult symptomatic asthmatic patients (n = 10 (4 women and 6 men), aged 23 - 60 years) were enclosed. It concerned atopic asthmatic patients selected on the basis of a positive screening result for specific IgE against inhalation allergens or a positive skin test with inhalation allergens. All individuals were newly diagnosed asthmatic patients. The study was double blinded with regard to laboratory parameters and lung function results. Patients were considered to be asthmatic when fulfilling the following criteria: reversible airway obstruction (increase in Forced Expiratory Volume in one second (FEV₁) after bronchodilator amounting to ≥12% predicted FEV₁) combined with PC20 < 2 mg/ml and/or a ≥10% decline in FEV₁ after an exercise of 6 - 8 minutes on a treadmill (EIB). Mean initial values for FEV₁, FEV₁/FVC, PC20, EIB, eosinophil count and serum ECP concentration were 71% of the predicted value, 0.64, 0.52 mg/ml, 37% decrease, 0.34 x 10⁹/l and 94 μg/l, respectively. Exclusion criteria comprised airway infection, acute or chronic inflammation different from asthma, autoimmune disease, neoplasma, cardiovascular disease and comedication with anticoagulants. After the first visit the patients received corticosteroids by inhalation (2 times a day budesonide 400 μg to 800 μg bd via Turbuhaler®) as well as β₂ mimetics (Formoterol®)
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or $\beta_2$ sympathicomimetica (Salbutamol®) as clinically indicated. Three patients started
with 2x 800 $\mu$g Budesonide and ended the study with 2x 400 $\mu$g Budesonide, while the
other patients were treated the whole study with 2x 400 $\mu$g Budesonide.
The reference group consisted of 223 apparently healthy adults (blood donors, 122
males, 101 females; aged 18 - 65 years).
The study was approved by the local medical ethical committee; patients who
participated signed an informed consent.

Study design
Patients did not receive inhaled or oral corticosteroids prior to the study medication
protocol. After the start of the study, the hospital was visited at 3, 6 and 9 months. At
each visit, blood sampling and lung function testing was performed. From subjects in the
reference group only blood samples were drawn. For measurement of CRP only 21
apparently healthy subjects were taken into consideration.

Laboratory tests
Blood samples were drawn in Vacutainer® tubes with K$_3$EDTA as an anticoagulant or in
tubes with addition of SST gel and clot activator (ref. 367652 and ref. 367783
respectively, Becton Dickinson, Plymouth, UK). In the anticoagulated blood samples
leukocyte count and differentiation was measured. Leukocyte counts and leukocyte
differentiation in blood samples were performed on a Sysmex NE-8000 hematology
analyzer (Charles Goffin Medical Systems BV, Tiel, The Netherlands).
Blood samples for serum preparation were drawn for measurement of ECP, total IgE and
CRP concentrations. In addition, at the first visit a blood sample was also drawn for
determination of specific IgE serum concentration against inhalation allergens. After
blood sampling, clotting occurred for 2 hours at 37°C. Thereafter, blood samples were
centrifuged for 10 minutes at 1350 x g. Subsequently, the serum samples were stored at
-20°C until analysis. ECP, IgE and specific IgE were measured with commercially
available immunoassays (Kabi Pharmacia, Uppsala, Sweden). C-Reactive Protein (CRP)
concentration was measured by nephelometry (Dade Behring, Marburg, Germany)

**Lung function tests**

Patients underwent spirometry by means of a master screen FRC (Jaeger Benelux bv, Wurzburg, Germany). In this way Forced Vital Capacity (FVC) and Forced Expiratory Volume (FEV$_1$) were assessed. FEV$_1$ is expressed as a percentage of the predicted value (FEV$_1$, % pred) and with respect to the Forced Vital Capacity (FEV$_1$/FVC); both measurements reflect airway obstruction. Bronchial responsiveness to inhaled histamine was assessed as described (20) by means of tidal breathing and doubling stepwise concentrations of histamine from 0.5 to 8 mg/ml. The histamine concentration causing a 20% fall in FEV$_1$ (PC20) was recorded after linear interpolation on a semi-logarithmic graph. Exercise-induced bronchoconstriction (EIB), an alternative parameter of airway inflammation, was assessed by exercising the patients on a treadmill for 6 - 8 minutes. FEV$_1$ was measured at 0.5, 5, 10, 15 and 20 minutes after starting the exercise. A decrease in FEV$_1$ of ≥10% was considered abnormal.

**Statistical analysis**

The statistical software package SPSS/PC, version +5.0 was used for statistical evaluation. Results are expressed as mean values together with the standard deviation. The statistical significance of deviations between groups was assessed by ANOVA analysis of variance and a T-test when appropriate. A paired T-test was assessed for statistical evaluation of patients results before and after therapy with inhaled corticosteroids. A p-value less than 0.05 was considered to be statistically significant.
ECP and eosinophil count in asthma

Results

Results are shown in figure 1. Compared with the reference group of apparently healthy individuals, asthmatic subjects before corticosteroid therapy showed significantly higher serum ECP concentrations and blood eosinophil counts (figure 1A, figure 1B). Three months after the start of therapy with inhaled corticosteroids these parameters did not show a statistically significant deviation any more in comparison with apparently healthy subjects. ECP per eosinophil (ECP/EOS) (figure 1C), granulocyte counts and CRP concentrations did not vary in time and were comparable with respect to apparently healthy subjects.

Immediately after the start of inhaled corticosteroids, asthma activity established by performance of lung function tests decreased; FEV₁ (% pred) (figure 1D), FEV₁/FVC and exercise-induced bronchoconstriction (EIB) (figure 1F) improved significantly, while PC20 (figure 1E) had improved significantly after 6 months of therapy.
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1B

1C

Eosinophil count (10^9/l)

month

ECP/Eosinophil (μg/10^9)

month

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1D

1E

115
Figure 1: Results of laboratory parameters and lung function tests before (month 0) and during therapy with inhaled corticosteroids (month 3, 6, 9) in 10 asthmatic patients. The thick line represents the mean longitudinal course of all patients. The two horizontal lines in figures 1A, 1B and 1C represent the 95% area of the reference group (n = 223). *X-axis statistically significant deviation of the patient group with regard to the reference group; X-axis statistically significant deviation with regard to month 0 (before start of therapy).

Both serum ECP concentrations and eosinophil blood counts correlated with lung function parameters that reflect the severity of airway inflammation (PC20 and EIB) (figure 2A, 2B, 2D, 2E). ECP/eosinophil ratios did not show a significant relationship with any of the lung function tests (figure 2C, 2F). We found a positive correlation between eosinophil counts and serum ECP concentrations (correlation coefficient 0.87,
Correlations between parameters reflecting lung obstruction (FEV₁ % pred, FEV₁/FVC) and laboratory test results were weak and in most cases not statistically significant. FEV₁ % pred, as a marker of broncho-obstruction, showed only a marginal correlation with the concentration of the inflammation marker CRP (r = -0.33, p = 0.04). FEV₁/FVC showed a weak but significant correlation with eosinophil counts and ECP concentrations (r = -0.38 p = 0.02 and r = -0.37 p = 0.02, respectively).

A wide range of overlapping results was observed between the serum ECP concentrations in the reference group and the individual results of the asthmatic patients (figure 1A). Similar overlap was seen between eosinophil blood counts in the reference group and the individual patient values (figure 1B).
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2B

Eosinophil Count (10^6/μl)

PC20 category

2C

ECP/Eosinophil (μg/ml)

PC20 category

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2D

![Graph showing the relationship between EIB (% decrease) and Eosinophil Cationic Protein (µg/µl). The graph indicates a positive correlation.]

2E

![Graph showing the relationship between EIB (% decrease) and Eosinophil count (10^9/µl). The graph shows a positive correlation.]

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Figure 2: Relationships between serum ECP, eosinophil blood counts and serum ECP/eosinophil ratios with PC20 (A,B,C) and Exercise Induced Bronchoconstriction (D,E,F) in asthmatic patients (n = 10) before and during corticosteroid therapy. Boxes indicate the 25th and 75th percentiles, with median values as horizontal lines indicated in the boxes. The length of the tail is a measure of the scattering of results.

a) \( y = -27x + 94; r = 0.46, p=0.003 \)
b) \( y = -0.09x + 0.31; r = 0.44, p=0.005 \)
c) no significant correlation

d) \( y = 1.8x + 29.2; r = 0.60, p<0.0001 \)
e) \( y = 0.007x + 0.069; r = 0.66, p<0.0001 \)
f) no significant correlation

PC20 category 1 = 0-0.5 mg/ml, PC20 category 2 = 0.51-1.0 mg/ml, PC20 category 3 = 1.1-2.0 mg/ml, PC20 category 4 = 2.1-4.0 mg/ml, PC20 category 5 = 4.1-8.0 mg/ml, PC20 category 6 = 8.1-16.0 mg/ml.
ECP and eosinophil count in asthma

Discussion

Prior to the start of therapy, serum ECP concentrations were significantly higher than during therapy. In several studies, serum ECP concentrations have been demonstrated to correlate positively with eosinophil blood counts (21, 22). In this study, a similar correlation between these parameters was established. We hypothesized that for determination of the disease activity grade it might be valuable to calculate the ECP release per eosinophil. However, the activity grade of the eosinophils expressed as ECP/Eosinophil ratio did not show any deviation if compared with the reference group. During therapy with corticosteroids, the ECP/Eosinophil ratio was stable. This observation means that corticosteroid treatment reduces the eosinophil blood concentration but does not influence the propensity of eosinophils to release ECP.

Like others (5, 6, 23), we found comparable relationships between eosinophil counts and ECP concentrations with hyperreactivity tests (PC20 and EIB). This observation implicates that serum ECP concentration does not yield any additional value over eosinophil blood counts to assess the effect of treatment with corticosteroids. This statement can be explained by the strong correlation between eosinophil counts in blood and ECP concentrations in serum, and the constant ECP/Eosinophil ratio in all subjects during the longitudinal study. To study the additional value of serum ECP concentrations in more detail it would be of interest when patients underwent an exacerbation. However, none of the patients in this study experienced an exacerbation of disease. The correlation coefficients between FEV₁ (%pred) and FEV₁/FVC with laboratory parameters are lower than in case of EIB and PC20. This finding can be explained by the fact that bronchus obstruction is a parameter in asthmatic patients that contributes to one of the criteria regarding asthma. Additionally, FEV₁ (%pred) and FEV₁/FVC improved up to three months with inhaled corticosteroids while PC20 and EIB improved continuously for over 9 months. Since serum ECP measurements are variable, they did not correlate with FEV₁ (%pred) or FEV₁/FVC, but they did with
The eosinophilic granulocyte is a major participant in airway disease and in the development of asthma (10, 12). In symptomatic patients, a local accumulation of eosinophils is found in the submucosa of the bronchi (3, 5). In agreement with these studies, we showed that asthmatic patients without steroid therapy had higher eosinophil blood concentrations in comparison with apparently healthy controls. These eosinophils are possibly activated to secrete more readily their stored products, e.g. ECP, which can be measured in serum (14). Various preanalytical conditions can influence the serum ECP concentration (21, 24, 25). In this study standardized preanalytical conditions have been applied for the release of ECP in serum (21, 22). A significant difference was detected between serum ECP from the patients' group and from the reference group. However, for individual cases, none of the laboratory parameters is of conclusive diagnostic value in detecting an asthmatic constitution. A wide range of overlapping results was observed between the reference group and the asthmatic patients.

As prevalence of asthma increases, the number of patients treated by physicians will rise in future. Assays for monitoring the activity status of the disease would be valuable. Treatment with corticosteroids and $\beta_2$ mimetics aims to reduce clinical symptoms by decreasing bronchial inflammation and improving bronchial tone (26, 27, 28). Effects can be registered by monitoring lung function parameters but also by follow-up from laboratory results. Because of the good correlation between eosinophils or ECP concentrations and hyperreactivity tests, we conclude that measuring these laboratory parameters may yield an alternative method to monitor effects of corticosteroids if lung function testing for hyperreactivity is not available. However, the additional value of serum ECP over eosinophil blood counts to detect the degree of severity in case of an asthmatic constitution for individual cases during treatment is doubtful.
References


