Eosinophil decranulation as an allergy activation marker
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Chapter 4

Eosinophil Cationic Protein in serum from nonatopic and asymptomatic atopic individuals after standardized blood clotting at 37°C

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Summary

Serum Eosinophil Cationic Protein (ECP) and serum ECP/Eosinophil ratio were measured in 223 apparently healthy individuals. The serum sample for ECP measurement was obtained by standardized clotting of the blood sample for 2 hours at 37°C. No statistically significant differences between men (n = 122) and women (n = 101) were found for either eosinophil blood count, serum ECP concentration or serum ECP/Eosinophil ratio. For serum ECP a reference range from 12-99 µg/l was established, whereas the serum ECP/Eosinophil ratio ranged from 61-367 µg ECP per 10⁹ eosinophils. Plotting serum ECP versus blood eosinophil count revealed a significant positive correlation (y = 141x + 18, Rsq = 0.45, p<0.001). The serum ECP/Eosinophil ratio, however, was found to decrease with increasing eosinophil blood counts.

Twenty-three percent of the apparently healthy individuals was found to have a positive score for IgE antibodies specific to inhalant allergens, and thus should be considered atopic. These atopic individuals demonstrated significantly higher mean serum ECP concentrations and eosinophil blood counts than did the nonatopic subgroup. In this healthy population, no decision level for either eosinophil blood count, serum ECP or serum ECP/Eosinophil ratio could be found that discriminated atopic from nonatopic individuals.
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Introduction

Initially, the eosinophilic granulocyte was considered to have an exclusively protective role, for example in host defense against parasites (1). Later, the eosinophil was recognized as a pro-inflammatory cell mediating allergic symptoms (2). Increased numbers of eosinophils may occur in blood and tissues of subjects with allergic diseases such as asthma, allergic rhinitis and atopic dermatitis. In most cases the concentration of eosinophils in affected tissues correlates with the severity of clinical symptoms (3, 4, 5). The eosinophilic granulocyte is involved in inflammation by release of granule proteins and inflammatory mediators (6). Eosinophil granule proteins are, amongst others, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil protein X (EPX) and eosinophil peroxidase (EPO). These granule proteins give rise to toxic effects on a target organism or on the surrounding tissue.

The mechanism of action, in particular the cytotoxic activity of eosinophil granule proteins, has been investigated already in detail. It has been hypothesized that ECP, being a highly cationic protein, facilitates channel formation in lipid membranes (2). This feature of ECP is different from other eosinophil granule proteins, such as MBP, which probably act by crosslinking membrane structures. ECP is considered to be one of the most cytotoxic granule proteins (7).

The mechanism by which ECP and other cationic granular proteins are released from eosinophils is poorly understood. With respect to clinical interpretation it is a drawback that publications on eosinophil-derived proteins in serum or lavage fluid either refer to essentially different sample processing procedures or do not yield any specific information with regard to preanalytical conditions.

Factors such as incubation time and temperature during sample processing have been demonstrated to be of significant importance for appropriate clinical interpretation of results (8, 9). Therefore, this paper describes the determination of reference values for ECP in serum, obtained under strictly defined clotting conditions, i.e. 2 hours at 37°C.
Moreover, the relation of serum ECP with eosinophil blood count, expressed as serum ECP/Eosinophil ratio, has been studied, in particular with respect to the detection of atopic individuals in an apparently healthy population.

**Subjects and methods**

Blood samples were drawn from 223 apparently healthy adults (blood donors; 122 males and 101 females; aged 18 - 65 years). Two serum specimens and one plasma sample (Vacutainer®, ref. 367703 SST with clot activator and ref. 367652 with K$_3$EDTA as an anticoagulant, Becton Dickinson, Plymouth, UK) were drawn from each subject. After venepuncture, blood samples were clotted immediately for serum preparation for 120 ± 10 minutes in a water bath of 37°C. After incubation, the samples were centrifuged during 10 minutes at 1350 x g at room temperature. Serum samples were stored at -20°C until the ECP concentration was assayed. Serum ECP concentrations were established by use of a radio-immuno-assay kit (Kabi Pharmacia, Uppsala, Sweden). Eosinophilic granulocytes were counted on a Sysmex NE-8000 hematology analyzer (Charles Goffin Medical Systems BV, Tiel, The Netherlands). Levels of IgE antibodies specific to inhalant allergens were estimated by means of a radio-immuno-assay kit (Phadiatop, Kabi Pharmacia, Uppsala, Sweden).

**Statistical methods**

For statistical analysis the computer program SPSS (Windows, release 6.1) was used. The T-test was applied to establish the statistical significance of differences in serum ECP concentrations, eosinophil blood counts and serum ECP/Eosinophil ratios between atopic and nonatopic individuals. P-values below 0.05 were considered to indicate
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statistical significance.
To determine reference ranges the 95% prediction intervals were calculated. Ranges for serum ECP and serum ECP/eosinophil ratio are shown in frequency distribution histograms. The reference ranges were determined as the estimation of the 2.5 and 97.5 percentiles directly. For this estimation the observations were ranked and a point was found below and above which 2.5% of the observations fall.
To establish the correlation between eosinophil blood count and serum ECP, and to indicate reference ranges for serum ECP concentrations in relationship with the eosinophilic granulocyte blood counts, regression analysis was applied and the 95% prediction intervals (with confidence of 95%) were calculated. After applying curve estimation the linear model seemed to be the best fitted curve; alternative models have lower Rsq values and are therefore less explanatory for the data.
For the correlation between the serum ECP/Eosinophil ratio and the eosinophil blood count, the same procedure as described for the reference range of serum ECP with respect to the eosinophil blood count was performed. However, for this parameter a cubic model was applied for drawing the best fitted curve.

Results

When comparing the results for male and female subjects, no statistically significant difference was detected in eosinophil blood counts. The mean value for both groups was $0.16 \times 10^9$ eosinophils/l, with a standard deviation (sd) of $0.10 \times 10^9$ eosinophils/l.
Similarly, serum ECP values were established for male and female subjects, with mean values $\pm$ sd of $40.1 \pm 19.5$ and $41.9 \pm 23.2 \mu g/l$, respectively. The serum ECP/Eosinophil ratios for males and females were $166 \pm 76$ and $183 \pm 83 \mu g/10^9$ cells respectively, a non-significant difference. Consequently, results obtained for men and women were combined to establish the following reference ranges: for serum ECP 12 - 99 $\mu$g/l and for serum ECP/Eosinophil ratio 61 - 367 $\mu$g ECP/ $10^9$ eosinophils (figure 1a + 1b).
Reference range of ECP in serum

**Figure 1:** Frequency distribution histograms for serum ECP concentrations (a) and serum ECP/Eosinophil ratios (b) in 223 apparently healthy adults.
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A significant increase in serum ECP results towards higher eosinophil blood counts was noticed (figure 2): $y = 141x + 18$, Rsq = 0.45 with $p<0.001$. In contrast, a statistically significant decrease in serum ECP/Eosinophil ratio towards higher eosinophil blood concentrations was observed (figure 3): $y = -1524x + 3772x^2 - 2960x^3 + 315$, Rsq = 0.28 with $p<0.02$.

The reference range corresponding with the 95% prediction interval for ECP concentrations with regard to the eosinophil blood count was found to be limited by the line functions: $y = 141x - 13$, $y = 141x + 49$ (figure 2).

The 95% prediction area for the serum ECP/Eosinophil ratio with respect to the eosinophil blood concentration was limited by $y = -1546x + 3939x^2 - 3286x^3 + 181$ and $y = -1501x + 3604x^2 - 2633x^3 + 449$ respectively (figure 3).
Figure 2: Serum ECP concentrations versus blood eosinophil counts in 223 subjects. Open circles indicate results from nonatopic individuals. Black circles reflect results corresponding with atopic subjects. Reference ranges with respect to the eosinophil count are limited by: $y = 141x - 13$ and $y = 141x + 49$. 
Figure 3: Serum ECP/eosinophil ratios versus blood eosinophil counts in 223 subjects. Black circles reflect atopic subjects, open circles correspond with non-atopic subjects. Regression analysis for determination of a bivariate reference area with respect to the eosinophil concentration resulted in the lines $y = -1546x + 3939x^2 - 3286x^3 + 181$ and $y = -1501x + 3604x^2 - 2633x^3 + 449$. 
Nobody in the randomly selected group of apparently healthy subjects (N = 223) suffered from obvious allergic complaints. Nevertheless, 23% of the subjects had a positive score for IgE antibodies specific to inhalant allergens. Thus, these subjects should be regarded as atopic.

In these atopic subjects significantly higher blood eosinophil concentrations and serum ECP concentrations were observed than in the nonatopic subjects (table I).

Table I: Mean values and standard deviations (sd) for eosinophil blood counts, serum ECP concentrations and serum ECP/eosinophil ratios in atopic (n = 52) and nonatopic (n = 171) individuals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>nonatopic subjects (mean value ± sd)</th>
<th>atopic subjects (mean value ± sd)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood eosinophils (10⁹/l)</td>
<td>0.15 ± 0.09</td>
<td>0.19 ± 0.12</td>
<td>p=0.02</td>
</tr>
<tr>
<td>serum ECP (µg/l)</td>
<td>39.1 ± 20.3</td>
<td>46.9 ± 23.2</td>
<td>p=0.02</td>
</tr>
<tr>
<td>serum ECP/EOS (µg/10⁹ cells)</td>
<td>174.7 ± 80.1</td>
<td>170.4 ± 77.7</td>
<td>p=0.73 (ns)</td>
</tr>
</tbody>
</table>

However, no statistically significant difference between the two subgroups was detected in serum ECP/Eosinophil ratios.

For individual, asymptomatic cases, serum ECP nor eosinophil blood count is of any value in predicting an atopic condition, as can be readily seen from the distribution of the observations from specific IgE-positive samples in figure 2 and 3.
Discussion

ECP is a granule protein that is released by the eosinophil in vivo in the process of an allergic reaction and in vitro during clotting of whole blood. Serum ECP might be useful as an indicator of allergic disease activity. The fact that the large majority of ECP measured in serum is in fact released during the clotting process, necessitates thorough standardization of the sample preparation. In the present study, whole blood samples were allowed to clot for exactly two hours at 37°C. This procedure resulted in a reference range for serum ECP from 12 - 99 μg/l, which is obviously higher than found in studies in which clotting took place at room temperature (10, 11). Another advantage is the better defined clotting at 37°C than at room temperature (8).

In particular in cases of eosinophilia the blood sample processing has a large influence on the assay results. Serum ECP concentrations demonstrated a positive correlation with blood eosinophil counts (figure 2). Therefore, we calculated the serum ECP/Eosinophil ratio in each individual sample, determined the reference interval of this ratio for the population investigated and plotted the ratio versus the eosinophil blood count. The relationship between serum ECP/Eosinophil ratio and blood eosinophil counts is important for determining the activation grade per eosinophil. Whereas serum ECP showed a positive correlation with eosinophil blood count, the serum ECP/Eosinophil ratio turned out to be negatively correlated with the eosinophil blood count. In particular at eosinophil blood counts below 0.2 x 10^9/l, a parabolic increase was apparent. An overall reference range from 61 - 367 μg/ 10^8 cells was calculated, and in figure 3 a 95% prediction area for the serum ECP/Eosinophil ratio with respect to the eosinophil concentration in blood is indicated. The decreasing trend of the serum ECP/Eosinophil ratio might amongst other factors be due to a relatively high release of ECP from persons with rather low eosinophil counts. Another explanation for the decreasing trend of serum ECP/Eosinophil ratio might be that in individuals with high eosinophil counts the turnover rate of the eosinophilic
granulocytes is higher compared with individuals with low eosinophil counts. In case of a high turnover rate, eosinophils in the circulation are relatively young cells, which might release a substantially smaller amount of ECP per eosinophil than relatively older cells.

One of the most typical cells characterizing atopic disease is the eosinophilic granulocyte (1). We demonstrated that in about 23% of apparently healthy subjects an atopic constitution was demonstrated on the basis of a positive score for IgE antibodies specific to inhalant allergens (12). Information concerning the frequency of positive scores for specific IgE to inhalant allergens in adult subjects populations is rare. Kerkhof et al. (13) reported that 32% of the Dutch population had detectable amounts of specific IgE antibodies to common aeroallergens, whereas in about 20% of a French population positive Phadiatop tests were observed (14).

In atopic subjects statistically significant higher serum ECP values and blood eosinophil concentrations were demonstrated in comparison with nonatopic individuals (table I). Detection of an increase in ECP serum concentrations in atopic individuals, who never had specific complaints due to allergic disease, is surprising. This observation can be explained by the fact that atopic individuals mostly reveal higher blood eosinophil concentrations (1). Nonatopic and atopic subjects showed similar results for serum ECP/Eosinophil ratios. From these observations it may be concluded that eosinophils of the atopic group of individuals are not enough preactivated to release an increased amount of ECP per eosinophil.

In accordance with results from other studies (15), we conclude that the sensitivity and specificity of eosinophil blood count, ECP serum concentrations and serum ECP/Eosinophil ratio are insufficient to detect an allergic constitution in an asymptomatic person. Therefore, an additional value of serum ECP or serum ECP/Eosinophil ratio over the simple determination of eosinophil blood count was detected. In our study, the atopic persons concern individuals in an inactive state of
allergy. Atopics in an active state of allergy are expected to release a more pronounced quantity of ECP per eosinophil. This consideration will be checked in a longitudinal study in individuals with eosinophil-related diseases.
References


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