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Chapter 6

Total amount of ECP per eosinophil as indicator for the activity state of eosinophils

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Accepted by Scand J Clin Lab Invest

Abstract

It is questionable whether Eosinophil Cationic Protein serum concentrations yield additional information regarding the activity of allergic diseases, because Eosinophil Cationic Protein serum concentrations correlate very well with blood eosinophil counts. We established spontaneous release of Eosinophil Cationic Protein from eosinophils during *in vitro* coagulation amounting to 0.03 - 0.5 pg per eosinophil. When comparing the serum Eosinophil Cationic Protein per eosinophil ratio with the eosinophil concentration, a decreasing trend of released ECP in serum was observed towards higher eosinophil counts. The total amount of *extractable* Eosinophil Cationic Protein in eosinophilic granulocytes was determined by means of extraction with 0.5% Cetyl-trimethyl-ammonium-bromide. The total amount of extractable Eosinophil Cationic Protein in eosinophils amounted to 0.4 - 17 pg/eosinophil. The results showed a slight tendency to decrease with higher blood eosinophil counts. Irrespective of their concentration in blood, eosinophils were demonstrated to release 2 - 4% of their total ECP amount. From experiments in this study it is concluded that ECP release during *in vitro* blood clotting or measuring the total amount of extractable ECP does not yield any additional diagnostic value to detect the degree of activation in eosinophils.

Introduction

During blood clotting, eosinophilic granulocytes become activated and release proteins in serum. The extent to which eosinophils release Eosinophil Cationic Protein (ECP) *in vitro* during clotting may reflect the state of activation of the eosinophil population *in vivo* (1). Due to *in vitro* clotting, pre-activated eosinophils might become activated *e.g.* by released platelet activating factor (PAF) or by activated complement components (2). As a result of blood clotting, proteins from the specific granules are released in serum. In healthy subjects, eosinophils are in majority not pre-activated. This statement implicates that eosinophils from healthy subjects will release lower amounts of granule proteins during *in vitro* clotting (1) in comparison with eosinophils from diseased subjects.

The extent to which eosinophils will be able to release ECP during *in vitro* clotting may reflect the overall state of activation of the eosinophil population *in vivo*. The higher the ECP level in serum, the higher will be the *in vivo* propensity of the eosinophils to release their content at the local site of inflammation. Indeed, in several groups of patients with allergic complaints an increased amount of circulating eosinophils has been detected (3). An increased concentration of ECP in serum may be due to an increased concentration of blood eosinophils instead of a higher state of activation of the eosinophil population *in vivo*. In several studies a statistically significant correlation between serum ECP and blood eosinophil count has been established (4, 5, 6, 7). Due to *in vitro* clotting the entire eosinophil population may be activated instead of a subgroup of pre-activated eosinophils. We assume that serum ECP possibly reflects the total number of eosinophils in a blood sample. However, if preactivated eosinophilic granulocytes from allergic subjects would release more ECP during *in vitro* clotting, serum ECP concentrations should be higher than expected from the blood eosinophil count. This is the reason why ECP serum concentrations should be evaluated with regard to the blood eosinophil counts. We additionally estimated the total amount of ECP in the eosinophils by

extraction. We investigated the possible additional diagnostic value of serum ECP per eosinophil and critical factors that may influence the procedure for measurement of this ratio and the clinical interpretation of the results.

Patients and methods

To establish the relationship between serum ECP concentration and eosinophil blood count, blood samples were drawn from 223 apparently healthy adults (blood donors; 122 males and 101 females; aged 18 - 65 years). One serum specimen and one anticoagulated blood sample (Vacutainer®, ref. 367703 SST with addition of clot activator and ref. 367652 with K_3EDTA as an anticoagulant, Becton Dickinson, Plymouth, UK) was drawn from every subject. After venepuncture, blood samples were clotted immediately for serum preparation during 120 ± 10 minutes in a waterbath 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 ECP concentrations were assayed. Serum ECP concentrations were established by application of a radio-immuno assay kit (Kabi Pharmacia, Uppsala, Sweden). Eosinophilic granulocytes were counted on a Sysmex NE 8000 Haematology Analyser (Charles Goffin Medical Systems BV, Tiel, The Netherlands).

The total amount of extractable ECP per eosinophil was established in a group of 40 apparently healthy blood donors. For determination of the total amount of ECP in eosinophils, a blood sample anticoagulated with EDTA was extracted with 0.5% CTAB (Cetyl-trimethyl-ammonium-bromide) for 10 minutes. After centrifugation at 8370 x g, ECP analysis was performed in the supernatant.
For additional investigations concerning dilution experiments, nineteen blood samples with eosinophil concentrations amounting from 0.21 to 0.71 x 10^9/l were diluted with

PBS two-, five-, ten- and twenty-fold, respectively. After dilution, the cell suspensions were lysed by addition of 0.5% CTAB.

Statistics

For statistical evaluation the computer program SPSS (Windows, release 6.1) was used. The statistical significance of differences in dilution steps was assessed by paired t-test analysis. P-values below 0.05 were considered to indicate statistically significant deviations.

Results

As shown in figure 1 a statistically significant linear relationship between serum ECP concentrations and blood eosinophil concentrations was established in healthy subjects ($y = 141x + 18$; $r = 0.67$, $p<0.001$).
When comparing the ECP release per eosinophil during *in vitro* clotting with the blood eosinophil concentration (figure 2) a decreasing trend towards higher blood eosinophil counts was demonstrated ($y = -1.5x + 3.8x^2 - 3.0x^3 + 0.3$; $r = 0.52$, $p<0.001$).
The total amount of ECP refers to the amount of ECP extracted from eosinophils with 0.5% CTAB. All 40 samples taken together, a mean total concentration of 5.4 pg ECP per eosinophil (range 0.4 - 17 pg/eosinophil, standard deviation 3.7 pg) was observed. In figure 3 significant lower amounts of extractable ECP per eosinophil are shown in samples with increasing eosinophil concentrations ($y = 0.82/x + 1.03$, $n = 40$, $r = 0.91$, $p<0.01$).

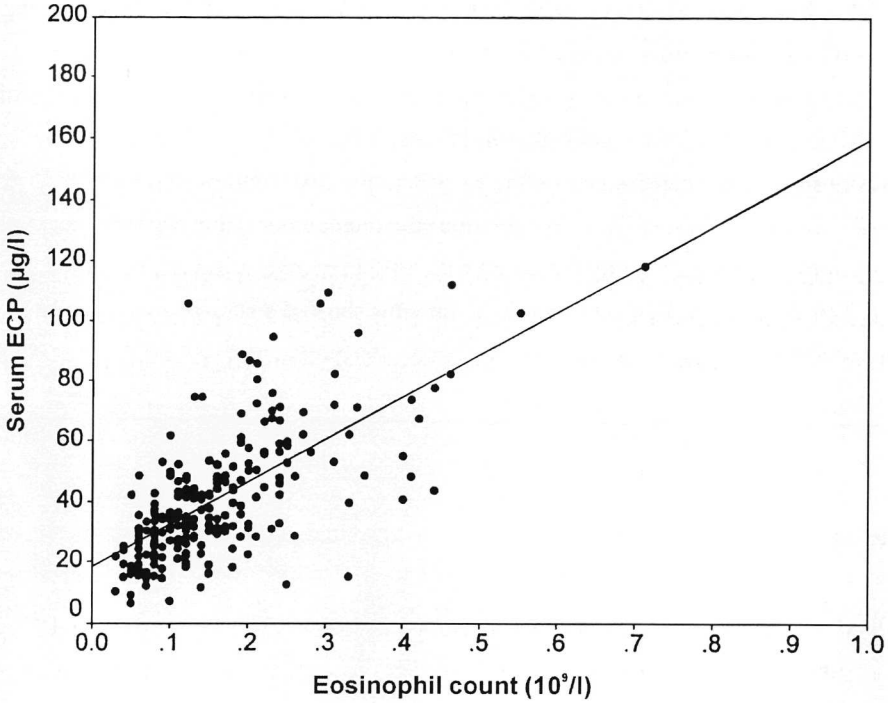

Figure 1: Relationship between serum ECP (y) and blood eosinophil count (x) in 223 apparently healthy adults; $y = 141x + 18$ ($r = 0.67$, $p<0.001$).

To examine whether the deviation from the straight line in figure 2 results from sticking of released ECP to membrane fragments, blood samples of 19 subjects were serially diluted, resulting in a wide range of eosinophilic granulocyte counts, and then lysed with CTAB. After evaluation of the total amount of extractable ECP per eosinophil, a statistically significant increase amounting to 16% in the first dilution step was observed. After the next step (five time dilution) the total amount of extractable ECP/Eosinophil was significantly increased with 56% in comparison with the initial amount. Towards higher dilutions, total ECP amounts showed a statistical significant increase of 28% (table I).

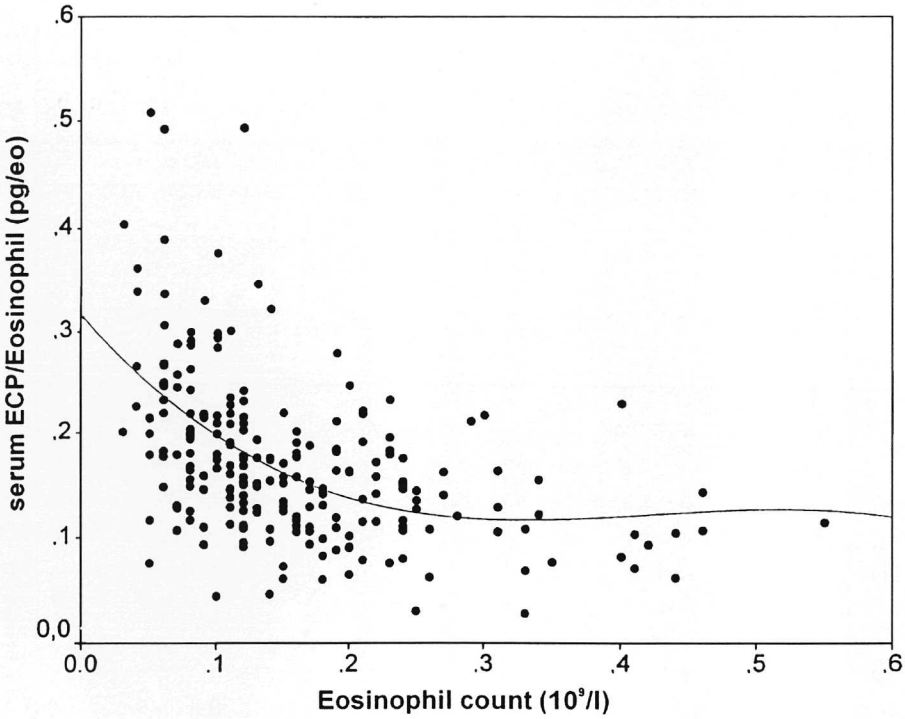

Figure 2: Relationship between ECP per eosinophil ratio released during in vitro clotting (y) and blood eosinophil count (x) in 223 apparently healthy adults;
$y = -1.5x + 3.8x^2 - 3.0x^3 + 0.3$ *(r = 0.53, p<0.001).*

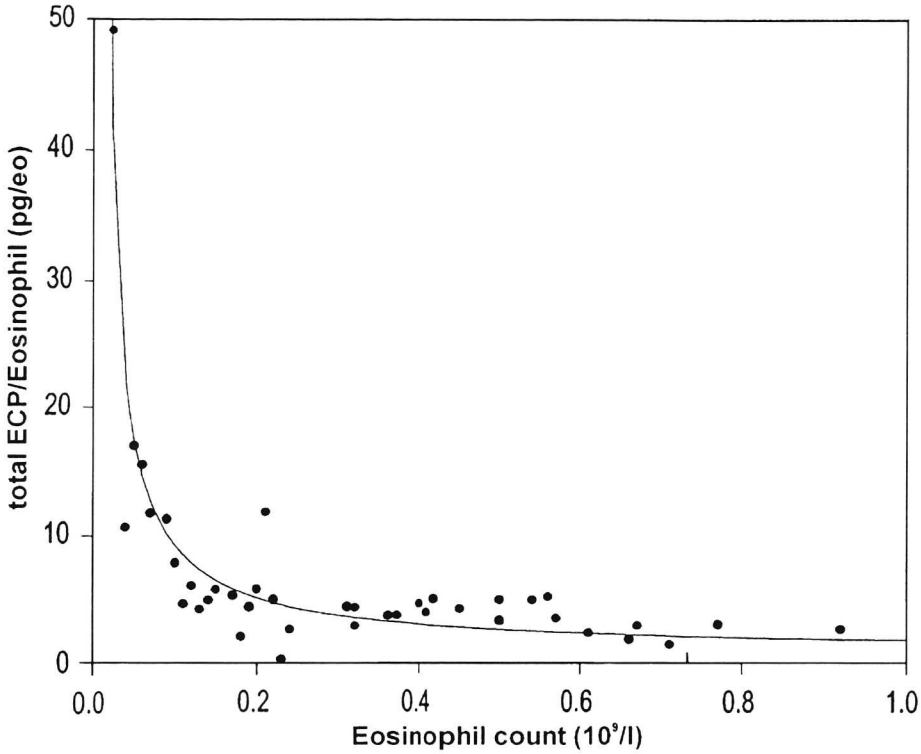

Figure 3: Relationship between total amount of extractable ECP per eosinophil (y) and the eosinophil count (x) in 40 individuals; $y = 0.82/x + 1.03$ ($r = 0.93$, $p<0.001$).

Table I: Total amount of ECP per eosinophil established in blood samples (n = 19) of several dilutions. Diluted samples were lysed with 0.5% CTAB. Percentage recovery was calculated in relation to the mean result in the undiluted samples. Undiluted eosinophil concentration was measured, other eosinophil concentrations were calculated by means of the dilution. * *statistically significant increase with respect to undiluted samples.*

Dilution	Eosinophil concentration (10^9/l) (mean ± sd)	total ECP/EOS (pg/eos) (mean ± sd)	% recovery
undiluted	0.48 ± 0.17	3.2 ± 1.3	100
1 : 1	0.24 ± 0.08	3.7 ± 1.4	116*
1 : 4	0.10 ± 0.03	5.0 ± 2.8	156*
1 : 9	0.05 ± 0.02	4.1 ± 1.9	128
1: 19	0.02 ± 0.01	4.1 ± 2.1	128*

Discussion

Specific granules in eosinophilic granulocytes contain basic proteins such as ECP (7). As a result of spontaneous clotting *in vitro*, eosinophilic granulocytes become activated, resulting in ECP release in serum (1). Quantification of ECP serum concentrations is considered to be an indicator of eosinophil activity (1, 9). However, serum levels of ECP do not adequately reflect *in vivo* release of ECP, because activation during *in vitro* clotting may result in essentially different ECP release. In addition, for proper evaluation it is important to standardize the preanalytical process for serum ECP determination (1, 7, 10, 11, 12).

A statistically significant relationship between serum ECP and blood eosinophil count has already been established previously (4, 5, 6, 7). Because of the relationship between

serum ECP and blood eosinophil count, also depicted in figure 1 of this study, the additional diagnostic value of serum ECP in comparison with blood eosinophil count is questionable. However, this statistically significant relationship between eosinophils and ECP demonstrated a range too wide for proper individual interpretation. Therefore, we estimated additionally the amount of serum ECP released per eosinophil. We and others hypothesized that the serum ECP per eosinophil ratio may yield additional information for determining eosinophil activity (12, 13). As a result of our study, serum ECP per eosinophil ratios were demonstrated to increase towards decreasing eosinophil blood counts. In previous studies, we demonstrated that patients with ulcerative colitis or allergic asthma showed similar relationships when comparing ECP per eosinophil release during *in vitro* clotting with eosinophil blood count (14, 15). The decreasing trend of the serum ECP/eosinophil ratio might amongst other factors be due to a relatively high release of ECP from individuals 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 may be 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.

Eosinophilic granules in subjects with lower eosinophil concentrations may contain a higher total amount of ECP. To investigate this hypothesis, the total amount of ECP was established by lysis with 0.5% CTAB. The total amount of extractable ECP per eosinophil demonstrated a slight tendency to increase in samples with decreasing eosinophil counts, from 0.4 to 17 pg per eosinophil. This observation is in agreement with the lower amount of ECP per eosinophil released during *in vitro* clotting in case of higher eosinophil counts. A coefficient of variation amounting to 10 percent of the total amount of extractable ECP per eosinophil between subjects was established. In previous experiments, the percentage release of granule proteins after activation with several

mediators has been quantified, but the total amount of extractable granule protein was not clearly indicated (16). Kita *et al.* (17) reported a range of 4.0 ± 1.2 (mean ± SEM) pg/eosinophil after lysis of the cells with 0.5% Nonidet P-40. Abu-Ghazaleh *et al.* (18) established a total amount of 5.3 ± 0.28 pg ECP/eosinophil after granule lysis in 0.01 M HCl (pH 2) and sonication. Carlson *et al.* (19) obtained 13.5 ± 2.0 pg ECP per eosinophil after extraction of eosinophils with 0.5% CTAB. Variability of ECP recovery after extraction in relation to the blood eosinophil concentration was not investigated. A deviation in the measurement of total amount of extractable ECP per eosinophil may be due to adhesion of the positively charged ECP to negatively charged membrane fragments. When determining the total amount of extractable ECP per eosinophil in different eosinophil dilutions in blood donors, a gradual increase in ECP recovery amounting to a maximum increase of 56% was observed. Therefore, the decreasing tendency of ECP per eosinophil released during *in vitro* clotting with higher eosinophil counts and the wide inter-donor variation in the total amount of extractable ECP per eosinophil can be only partly due to this artefact. In the present study the range in the total amount of extractable ECP per eosinophil between low-level and high-level eosinophil counts is 35 times (0.4 pg ECP/eosinophil in comparison to 17 pg ECP/eosinophil), and the ratio of ECP released per eosinophil during *in vitro* clotting showed a difference between low and high levels of approximately 6 times (0.06 pg ECP/eosinophil in comparison to 0.36 pg ECP/eosinophil). When blood eosinophil counts are decreased beyond the reference range (0.1 x 10^9/l) the release of ECP during *in vitro* clotting is about 0.1-0.5 pg/ eosinophil, from cells with a total amount of 10 to 15 pg ECP/eosinophil. Thus, during *in vitro* clotting, about 2% of the total amount of ECP is released in serum. A similar range was also established for higher concentrations of eosinophils, *i.e.* when the eosinophil count amounted to 0.4 x 10^9/l, the releasability of ECP during *in vitro* clotting was 0.05 to 0.2 pg/ eosinophil. With a total amount of 2-5 pg ECP/eosinophil, the percentage release of ECP in this case was about 3%. Thus, no deviation in percentage release of ECP during *in vitro* clotting with respect to eosinophil count was detected. We conclude that eosinophils, independent of their

number in blood, release approximately identical, relative small amounts of their total ECP content during *in vitro* clotting. Therefore, the hypothesis that priming factors that are present in allergic patients, preactivate eosinophils *in vivo* both locally and systemically (1, 2) is not confirmed by the results of our study.

We conclude that measurements of ECP release during *in vitro* clotting or of the total amount of extractable ECP do not yield additional information to detect eosinophil activity in healthy subjects with various eosinophil concentrations. Therefore, it is not possible to establish the activation stage of eosinophils with one single test.

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