Chapter 3

Eosinophil Protein X concentration in serum is dependent on blood eosinophil concentration

C. J. Pronk-Admiraal and P.C.M. Bartels
Department of Clinical Chemistry, Haematology and Immunology, Medical Centre
Alkmaar, The Netherlands

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Summary

The relationship between the blood eosinophil concentration and the serum Eosinophil Protein X concentration was investigated in 80 individuals. Higher blood eosinophil counts resulted in clearly increased serum Eosinophil Protein X concentrations. However, the amount of Eosinophil Protein X released per eosinophilic granulocyte was significantly higher in individuals with lower blood eosinophil counts.
Atopic individuals (N = 19) showed a significantly higher blood eosinophil concentration ($p = 0.002$) and serum Eosinophil Protein X concentration ($p = 0.004$) and a significantly lower serum Eosinophil Protein X/eosinophil ratio ($p = 0.02$), compared to non-atopic individuals (N = 61). However, the concentration of Eosinophil Protein X in serum from atopic and non-atopic individuals seems not to be different if the blood eosinophil concentration is taken into account. When considering serum Eosinophil Protein X concentration as an indicator of eosinophil activation, for instance in asthmatic patients, the blood eosinophil count should be considered for correct clinical interpretation of results.

Introduction

Eosinophils play an important role in the immune response to parasites and in the pathogenesis of certain inflammatory diseases, especially asthma. The granules of the eosinophilic granulocytes contain a number of highly cationic proteins, which are released following activation and stimulation of the eosinophil. Release of granule proteins from eosinophils is a selective phenomenon with respect to the individual proteins (1). Several proteins have been characterized, including eosinophil cationic protein (ECP) and Eosinophil Protein X (EPX), which is synonymous with eosinophil-derived neurotoxin (EDN) (2). ECP and EPX are single-chain proteins with a molecular mass in the range of 18 - 21 kDa, and pI>11 and pI>9, respectively (2). The measurement of eosinophil-derived proteins such as EPX in biological fluids may be a useful indicator of eosinophil activation.
EPX has a lower pI than ECP. This causes EPX to stick less easily to cell membranes after degranulation than ECP. Therefore, it might be a more suitable marker to measure the state of activation of the eosinophil.

Reported ranges of EPX concentrations in serum deviate widely between laboratories (3, 4). In the measurement of eosinophil-derived proteins in serum it is particularly important to standardize the handling of the blood samples (7, 8, 9, 10). Previous studies have shown that serum concentrations of eosinophil granule proteins are related to the severity of asthma (5, 6) and may provide an additional tool to monitor the efficacy of anti-inflammatory therapy.
In our study, the relationship was established between blood eosinophil counts and the serum EPX concentration under standardized conditions. Furthermore, atopic and non-atopic individuals within this group were compared to establish differences in blood eosinophil counts, serum EPX concentration and serum EPX concentration recorded per eosinophil.

Materials and Methods

Blood samples were taken from 80 apparently healthy adults (males and females, aged 18 - 60 year). From each subject two serum samples (Vacutainer®, ref. 367783, with addition of SST gel and clot activator, Becton Dickinson, Plymouth, UK) and one plasma sample (Vacutainer®, ref. 367652, with addition of K_3EDTA as an anti-coagulant, Becton Dickinson, Plymouth, UK) were obtained. After venepuncture, blood samples were clotted for 60 ± 10 minutes in a waterbath of 37°C. After clotting, blood samples were centrifuged at room temperature for 10 minutes at 1350 x g. Subsequently, the serum samples were stored at -20°C until the serum EPX concentration was measured. Serum EPX concentrations were established with a radio-immuno-assay kit (Kabi Pharmacia, Uppsala, Sweden), with a interassay coefficient of variation of 4.8 - 10.6% as specified by the firm. This range has been confirmed by our own experiments. For the EPX concentration a detection limit of 3 µg/l has been established. Eosinophil concentrations in blood samples were determined with a Sysmex NE-8000 hematology analyzer (Charles Goffin Medical Systems BV, Tiel, The Netherlands). IgE concentrations and determination of IgE antibodies specific to inhalant allergens were measured with a radio-immuno-assay kit (Total IgE and Phadiatop; Kabi Pharmacia, Uppsala, Sweden).

Statistics

The statistical significance of the regression coefficient showing the correlation between blood eosinophil counts and serum EPX concentrations was established by application of multiple regression analysis. To establish statistically significant differences between the subject groups on the basis of blood eosinophil concentrations, the Mann-Whitney-U test was applied.

This test was also used to determine statistical differences between atopic and non-atopic individuals.

Results

Serum EPX concentrations obtained at different blood eosinophil counts are depicted in figure 1. It is apparent from this figure, that the serum EPX concentration increased significantly with higher blood eosinophil counts.

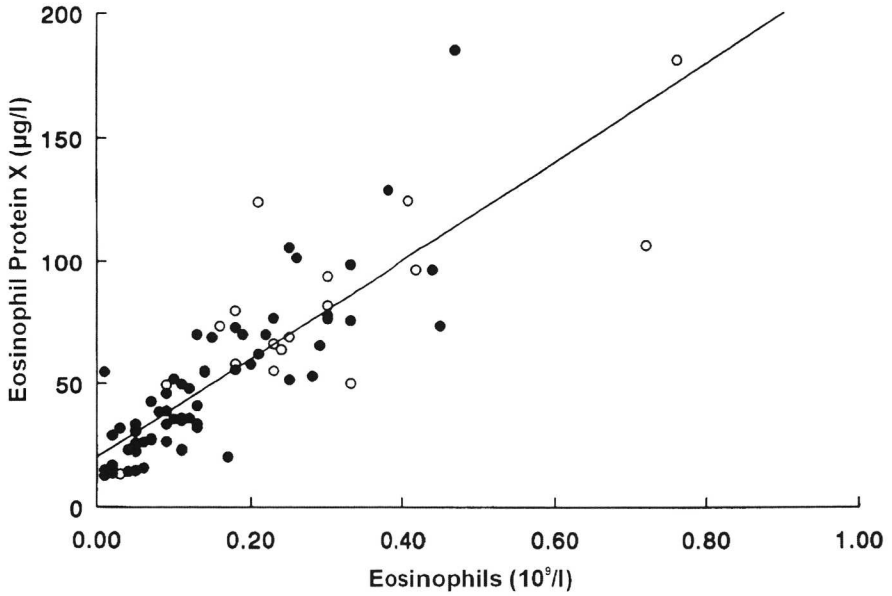

Figure 1: EPX concentrations determined in samples clotted at 37°C versus eosinophilic granulocyte blood counts in 80 individuals. Black circles represent values from samples of non-atopic individuals. Open circles show results from samples of atopic individuals. Results of statistical analysis: $y = 200x + 20$, $r = 0.84$ (37°C), $p<0.001$.

Subsequently, the mean quantity of EPX released per eosinophil was calculated. The whole group (N = 80) was divided in four groups with blood eosinophil counts of 0 - 0.10 x10^9/l (N = 29), 0.11 - 0.20 x10^9/l (N = 23), 0.21 - 0.30 x10^9/l (N = 17) and 0.31 - 0.80 x10^9/l (N = 11). For each group the median of the serum EPX/eosinophil ratio was calculated. Results are depicted in figure 2. A statistically significant increase was found in the EPX/eosinophil ratio in subjects with lower blood eosinophil counts.

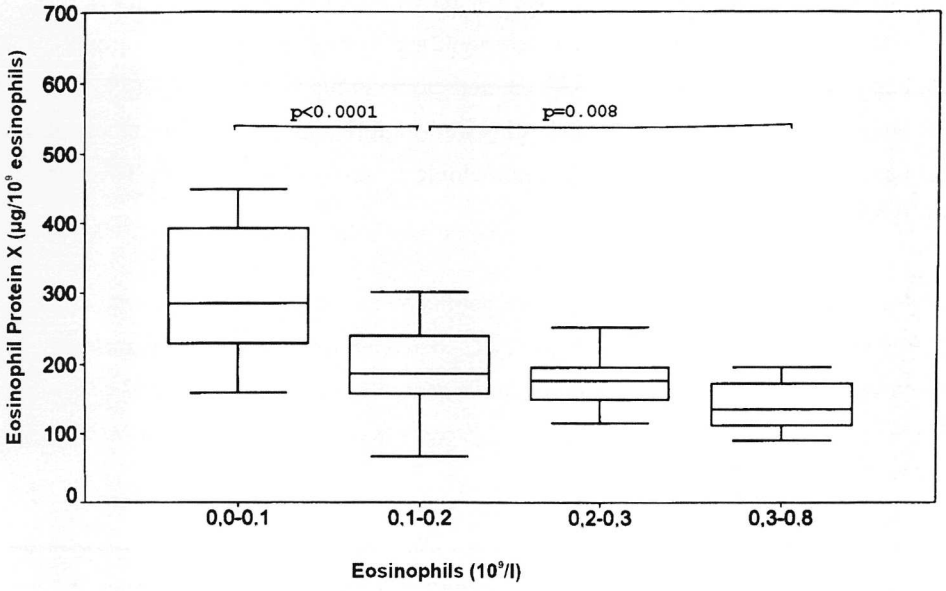

Figure 2: Median values (horizontal line inside the box) concerning serum EPX/eosinophil ratio in 4 groups with regard to different ranges for eosinophil blood counts. Boxes indicate the 25th and 75th percentiles, and the length of the tail, which is a measure for scattering of results, is shown by the upper and lower lines.

Total IgE concentrations and concentrations of IgE specific to inhalant allergens were determined in the serum samples from 80 apparently healthy individuals. An individual is considered to be atopic when the IgE concentration is above 120 kIU/l (11, 12) and the result for IgE specific for inhalant allergens is positive. Within the 80 adults, who were all without allergic complaints, 19 (25%) were classified to be atopic by application of the criteria mentioned above. The blood eosinophil count, serum EPX concentration and serum EPX/eosinophil ratio of both groups are reported in table I. The blood eosinophil counts were significantly higher in the atopic individuals (median = 0.23 x 10^9/l) than in the non-atopic individuals (median = 0.11 x 10^9/l, p=0.002). Serum EPX concentrations were significantly higher in the atopic group (median = 69 µg/l) in comparison with the non-atopic group (median = 41 µg/l, p=0.004). The serum EPX/eosinophil ratios were significantly lower in the atopic group (median = 165 µg/10^9 cells) than in the non-atopic group (median = 214 µg/10^9 cells, p=0.02).

Table 1 : Median values of the established parameters in atopic and non-atopic subjects. Results of application of the Mann-Whitney U- Wilcoxon Rank Sum test, to establish statistically significant differences between the groups of atopic (N = 19) and non-atopic (N = 61) subjects. A statistical significant difference is considered to be present when p-value<0.05.

Parameter	p-value	median (atopic)	median (non-atopic)
blood eosinophil count (10^9/l)	0.002	0.23	0.11
serum Eosinophil Protein X (µg/l)	0.004	69	41
serum Eosinophil Protein X/ eosinophil (µg/10^9 cells)	0.02	165	214

Discussion

The EPX content in eosinophilic granulocytes is estimated to be about 17 µg/10^6 eosinophils (13). When considering this estimation in combination with the results from our study, it should be emphasized that the release of EPX in serum during clotting at 37°C amounts to 0.3 to 2% of the total EPX content in eosinophils. As a consequence, the amount of actually *in vitro* released EPX is only a small fraction of the total content of EPX in eosinophils. A minimal fraction of the EPX concentration in serum might be due to release from neutrophilic granulocytes. Compared with eosinophils, EPX is present in neutrophilic granulocytes to a very small extent, *i.e.* 1 - 2% of the content of an eosinophilic granulocyte (14).

Compared with EPX, even less ECP is being released from eosinophils *in vitro* (9, 10). The various mechanisms by which eosinophils may be activated and release their granule proteins are only poorly understood. The reason for deviations between ECP and EPX release has not yet been elucidated. One possible explanation may be the fact that ECP will tend to stick more tightly to cell membranes than EPX because of the more basic character of ECP. Another explanation is the selective release mechanism of both granule proteins (1). Differences in mechanisms and kinetics for clearing these proteins from the circulation should also be taken into consideration.
It has been demonstrated that ECP has the ability to bind to clotting factors (15). Particular information concerning the interaction between clotting and EPX release after blood sampling is not yet available.
Because of the striking effect of the temperature of serum preparation on *in vitro* release of granule proteins (7, 8, 9, 10), blood samples should be clotted in our opinion at a precisely fixed temperature. We prefer to clot blood samples at 37°C. This temperature will yield a rather extended range of EPX concentrations in samples from different individuals, which are still within the detection range of the kit.
In a previous study it has been stated that eosinophils of allergic patients excrete EPX

more easily than do eosinophils of healthy persons (13). Concerning this statement, it may be of clinical importance to calculate the ratio of serum EPX concentration to the blood eosinophil count. When calculating this ratio for apparently healthy individuals, we observed that the amount of EPX released per eosinophil demonstrated a significant tendency to increase with decreasing blood eosinophil counts. An explanation for this observation might be that eosinophils in samples with a rather high number of eosinophils are less primed than eosinophils in samples with lower eosinophil counts.

Concerning the laboratory procedure for measurement, the relatively high serum EPX/eosinophil ratio in the lower blood eosinophil concentration range might be caused by inaccuracy in the eosinophil count measurement. However, experiments dealing with dilution of the blood samples did not yield any indication with respect to a systematic deviation in the low blood eosinophil count range.
Both the blood eosinophil count and the serum EPX concentration are significantly higher in the atopic group. Finally, the serum EPX/eosinophil ratio is significantly lower in the atopic group than in the non-atopic group.

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