

Original article

Measurements of ECP in serum and the impact of plasma coagulation

Serum measurement of ECP (eosinophil cationic protein) is used as an indication of eosinophil activation in diseases such as asthma. The levels are dependent on sample handling, since a certain amount of ECP is released during storage. The mechanisms that induce this *in vitro* release are not known, but are supposed to be related to the coagulation process. The aim of this study was to investigate this further. ECP was measured in EDTA plasma and serum at 22 and 37°C from healthy individuals and patients with asthma and allergy. The serum levels of ECP increased with temperature. Recalcification of citrated plasma in the presence of granulocytes with increasing concentrations of Ca^{2+} showed a dissociation between the levels of ECP and the occurrence of coagulation. Further experiments indicated that plasma coagulation is not of any importance for the degranulation of eosinophils, nor did the addition of platelets or mononuclear cells affect the ECP levels. Incubations of granulocytes with fresh or frozen plasma and Ca^{2+} suggested the existence of a freezing labile factor in plasma, necessary for the degranulation of healthy eosinophils, but not for allergic/asthmatic eosinophils. Further experiments with pure eosinophils indicated the existence of factors in serum and plasma which facilitate ECP secretion of an active, temperature-dependent nature. We conclude that the raised ECP levels in serum, as compared to EDTA plasma, are unrelated to the coagulation process, but are due to the continuous secretion *ex vivo* of ECP from active eosinophils. This process is time and temperature dependent and may be facilitated by eosinophil-activating components in the extracellular environment.

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ECP (eosinophil cationic protein) is a zinc-containing, extremely basic protein, contained in the peroxidase-positive and -negative granules of the eosinophilic granulocytes (1–4). ECP occurs in at least three different molecular weight forms, caused by differences in glycosylation of the protein (1). It is a strongly cytotoxic protein, and is probably important for defense against parasites. ECP binds to heparin and interacts with proteins in blood coagulation. ECP occurs in serum both as a free protein and in complex with α_2 -macroglobulin, which binds to ECP noncovalently.

Previous studies have shown that measurements of ECP may be used as markers of eosinophil activity (5–8), and that the eosinophilic granulocyte plays a major role in the development of asthma and allergy, as well as in rheumatoid arthritis, psoriasis, and adult celiac disease (9). Studies have also shown that

lymphocytes and eosinophils apparently interact during the development of asthma (8), and a predominance of CD4+ lymphocytes has been found in the bronchial mucosa of asthma patients. One of the cytokines secreted by these lymphocytes is IL-5, which selectively promotes the differentiation, priming, and survival of eosinophils *in vitro*, as well as enhancing the adhesion of eosinophils to vascular endothelial cells (8). Measurements of IL-5 in patients have shown increased levels, correlating with the activity of asthma.

A number of studies on the clinical value of serum ECP in asthma have been performed (5–8, 10–12). Serum ECP was shown to be related to asthma severity as measured by FEV₁ and histamine PC₂₀. The elevated levels of ECP may help to identify patients with chronic asthma and at risk of inflammatory exacerbation. The higher values of serum ECP in patients with asthma and allergy are believed to be caused by the higher intrinsic activity of the eosinophilic granulocytes of these patients, as compared with healthy individuals.

Measurements of ECP in plasma and serum have

Abbreviations. ECP: eosinophil cationic protein; EDTA: ethylene diamine tetraacetic acid; MACS: magnetic cell separation system; PBS: phosphate-buffered saline; CD: cluster of differentiation; IL: interleukin.

shown that the values in EDTA plasma are much lower than the ones in serum. The ECP levels in serum have also been shown to be dependent on both the time and the temperature at which the blood samples have been stored before centrifugation (13). The reason that EDTA plasma levels are so much lower than serum levels, for both healthy and allergic/asthmatic individuals, has not been explained. Some authors have assumed that this difference is due to the coagulation process, which has been believed to stimulate the eosinophils to secrete ECP.

The aim of this study was to investigate the impact of the coagulation process on the serum levels in experiments with granulocytes and plasma obtained from healthy subjects, patients with active asthma, and patients with allergy.

Material and methods

For the purpose of measuring ECP in serum and plasma at 22 and 37°C, whole blood without anticoagulants and EDTA or citrated blood was taken, after informed consent, from 24 healthy donors, eight patients with acute asthma, and four patients with allergy. The asthma patients had strong obstructive symptoms, had no oral steroid medication, and took not more than 400 µg of inhaled budesonide daily. The allergic patients had ongoing symptoms of rhinoconjunctivitis or skin symptoms, and had positive results for Phadiatop. The blood was incubated for 1 h at 22 or 37°C. After this, serum and plasma were obtained by centrifugation of the blood at 1835 g. The plasma and sera obtained were stored at -70°C until measurement of ECP levels.

For the purpose of purifying granulocytes, heparinized or citrated blood was collected from healthy patients and allergic patients. The allergic patients in these experiments all had ongoing symptoms of rhinoconjunctivitis or skin symptoms and had positive tests for Phadiatop.

In an experiment with granulocytes from healthy individuals and plasma from allergic patients, the plasma was used from 10 patients with ongoing symptoms of asthma and allergy. All of these patients had the diagnosis of asthma, and four of them also had positive Phadiatop tests.

Granulocytes were purified with dextran sedimentation or Percoll purification of heparinized or citrated blood (14, 15). Mononuclear cells and eosinophilic granulocytes were also obtained by Percoll purification, followed by (in the case of eosinophils) the process of the magnetic cell separation system (MACS) (16). This separates the eosinophils from the neutrophils by antibodies against CD16, which bind to the neutrophils, followed by passing the granulocytes through a magnetic field. After purification, the granulocytes

were suspended in PBS (phosphate-buffered saline) at a concentration of $10^{10}/l$. The median purity of eosinophils after MACS separation was 97% (range 84.8–99.6%).

In the plasma-granulocyte system, equal parts of granulocyte suspension and plasma were mixed, so that the final concentration of granulocytes was $5 \times 10^9/l$. In the experiments with purified eosinophils, the final concentration was $0.5 \times 10^9/l$. The final concentration of mononuclear cells was $3 \times 10^9/l$.

The reproducibility of the procedure of recalcifying plasma in the presence of purified cells was evaluated in three experiments in which plasma and purified granulocytes from the same donor were mixed at room temperature in 8–10 different samples, recalcified, and centrifuged after 1 h. The mean CV from 28 samples was found to be 13.4% (range 10.1–16.7%).

The possibility of activation of the eosinophils in the granulocyte-plasma system was investigated by three experiments in which serum-opsonized Sephadex particles (17) were incubated with granulocytes mixed with citrated plasma or plasma and Ca.

Platelet-rich and platelet-poor plasma was obtained after centrifugation of citrated blood at 181 and 1835 g, respectively.

A 100 mM CaCl_2 solution was obtained by mixing CaCl_2 (Merck) with mQ water (Millipore, Millipore Corporation, USA). The concentration was checked with atomic absorption spectroscopy.

Pharmacia's ECP-RIA was used to measure ECP levels (2).

Statistical methods

Mann-Whitney's nonparametric test for unpaired samples and Wilcoxon's nonparametric test for paired samples were used to estimate significant differences. Linear correlations were calculated with the computer program STATISTICA for Windows (Statsoft, Tulsa, OK, USA).

Results

When the ECP levels were measured in the EDTA plasma and serum samples from healthy, allergic, and asthmatic patients, the following results were seen. In plasma, no difference with temperature or between the different groups of donors was seen. Therefore, only values for 22°C are shown. As to the serum values, different patterns were seen for the different groups of donors, as well as for different temperatures. For healthy donors, the values at 22°C were, on average, 7 µg/l and were all below 14 µg/l, and the values at 37°C were usually two to four times the value at 22°C. For asthmatic and allergic donors, the serum values varied

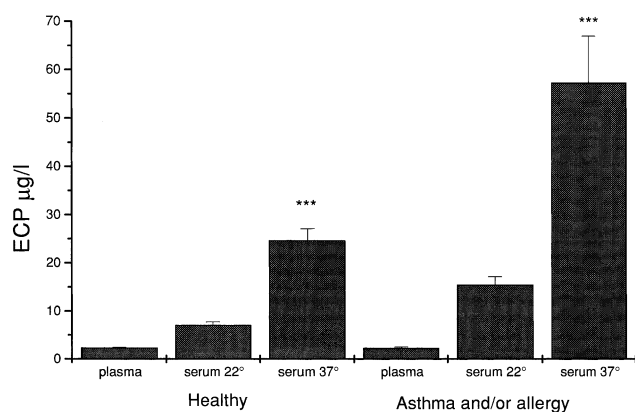


Figure 1. Levels of ECP in plasma and serum at 22 and 37°C. Blood was obtained from 22 healthy persons and 12 patients with acute asthma or allergy. Results are presented as mean values and standard error of mean (SEM). *P* values (***) indicate differences between sera obtained at 22 and 37°C (Wilcoxon's nonparametric test for paired samples).

more and were higher at both 22 and 37°C, as compared to healthy donors ($P < 0.001$ for both temperatures). At 37°C, values as high as 123 g/l were seen (Fig. 1).

For both healthy and allergic/asthmatic donors, the values in serum at 37°C were significantly higher than those at 22°C ($P < 0.001$) (Wilcoxon's nonparametric test for paired samples). The relative increase was similar in the two groups.

Correlations between values at 22 and 37°C are shown in Fig. 2. The correlation was higher ($r = 0.68$) for the allergic/asthmatic group than for the healthy donors ($r = 0.58$). For all donors, $r = 0.78$.

In order to assess the possible stimulatory role of coagulation on ECP secretion, granulocytes (purified with Dextran) from six healthy and four allergic donors were mixed with citrated plasma and recalcified with concentrations of Ca from 0 to 10 mM, followed by incubation for 1 h at 22°C. The ECP levels in the samples did not significantly increase (compared with the samples without Ca) for any of the Ca concentrations used, either for nonallergic or for allergic donors. Coagulation of the samples occurred at a Ca concentration of 2.5 mM (and higher), but did not significantly increase the levels, as compared to the noncoagulated samples (Fig. 3). Similar results were obtained when the experiments were performed at 37°C.

The possible stimulatory effect of different environments on purified eosinophilic granulocytes was also studied. In seven separate experiments, eosinophils from healthy donors (one in each experiment) were mixed with buffer, an albumin solution (38 g/l), serum, or citrated plasma (from the same donor). The samples were kept at 22 or 37°C for 1 h. The results (Fig. 4) show that when the eosinophils were mixed with serum or plasma, the ECP secretion was significantly higher ($P < 0.05$) than that seen in the albumin solution at both temperatures. However, no differences were seen

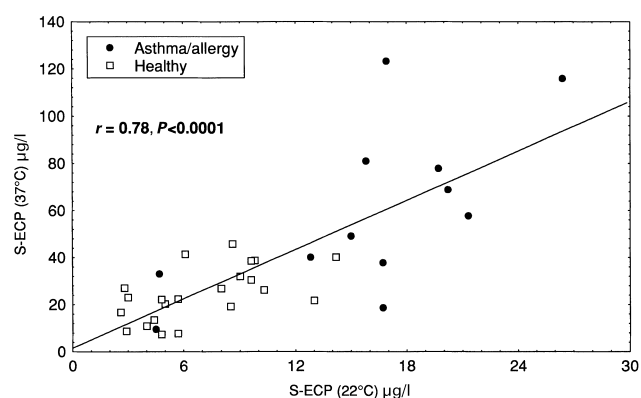


Figure 2. Correlation between serum measurements of ECP at 22 and 37°C for healthy and allergic/asthmatic donors (same patients as in Fig. 1). That serum levels were more dependent on temperature for asthmatic/allergic group than for healthy group indicates that ECP levels depend not only on eosinophil count, but also on other factors, such as eosinophilic activation.

between eosinophils suspended in buffer or albumin solution (results calculated after subtraction of the initial ECP values of the plasmas and serums used).

For each environment (buffer, albumin, serum, or plasma), the ECP secretion increased significantly with temperature, indicating that the secretion was of an active kind. The results indicate that coagulation is not essential for ECP secretion, since serum contains products originating from coagulation, but plasma does not.

Granulocytes from healthy and allergic or asthmatic donors were mixed with fresh or frozen citrated plasma, recalcified, and stored at 22 or 37°C followed by centrifugation after 1 h. After measurement of ECP levels, the following was observed. When healthy and allergic granulocytes were mixed with fresh or frozen plasma, the healthy and allergic granulocytes behaved differently with respect to temperature dependence and type of plasma used. For healthy granulocytes, there was a significant, pairwise temperature difference only when fresh plasma was used. For allergic/asthmatic granulocytes, on the other hand, there was a significant temperature difference (pairwise) irrespective of which kind of plasma was used (Fig. 5). As mentioned earlier, the plasma levels of ECP were always below 14 µg/l.

In order to study the possible contribution of other cells to the ECP release after coagulation, granulocytes from 10 healthy and seven allergic donors, purified with Percoll, were mixed with their own platelet-rich or platelet-depleted plasma and/or their own mononuclear cells and kept for 1 h at room temperature and at 37°C after recalcification. No significant rise in ECP levels was seen at either 22 or 37°C when platelets and/or mononuclear cells were added to the granulocytes and plasma for healthy donors. In the case of allergic donors, there was an increase ($P < 0.05$) when platelets were added at 22°C; otherwise, the addition of other cell components was of no importance (Fig. 6).

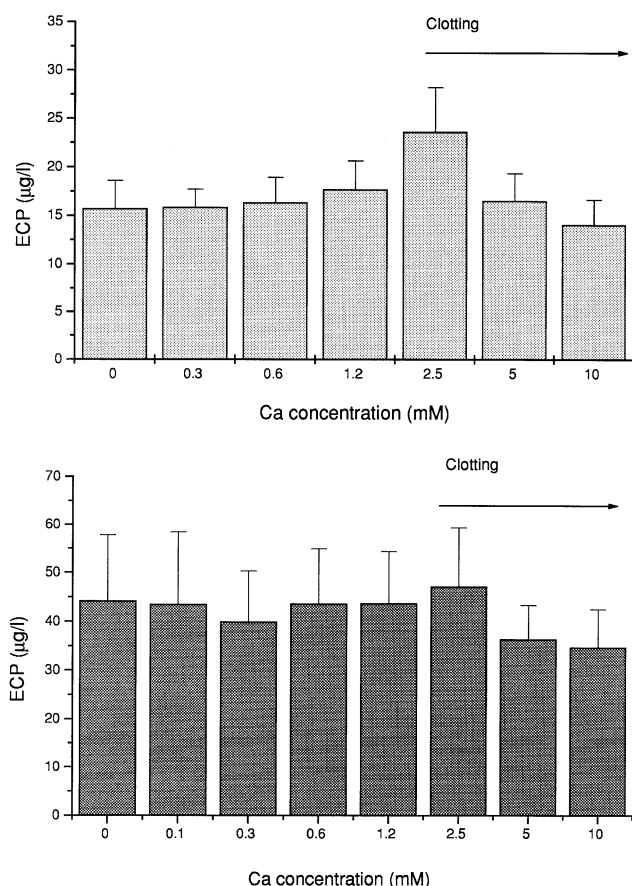


Figure 3. Eosinophilic activation in experiments with granulocytes, citrated plasma, and Ca in different concentrations, incubated at 22°C for 1 h. Blood from six healthy and four allergic blood donors was used. In each experiment, granulocytes and plasma from same donor were used. Coagulation occurred at Ca concentrations of 2.5 mM and above. No significant increase of ECP secretion was seen at those Ca concentrations (2.5 mmol/l and higher) where coagulation occurred, for either healthy or allergic blood donors. Means and SEM. Upper graph: healthy donors; lower graph: allergic donors.

In the experiment with serum-opsonized Sephadex particles, the levels of ECP after 1 h at room temperature, followed by centrifugation, were two to three times higher in the cell supernatants after exposure of cells with particles than in control samples with granulocytes and plasma, as well as granulocytes, plasma, and Ca. This experiment indicates that the effect of a Ca-chelation, such as citrate, on eosinophil degranulation is reversible.

Discussion

The temperature dependence of ECP in serum has already been studied for normal donors (13), and it has been shown that the ECP secretion increases with the temperature in which the blood samples have been stored before centrifugation. Our results show that for patients with asthma or allergy, the increase with

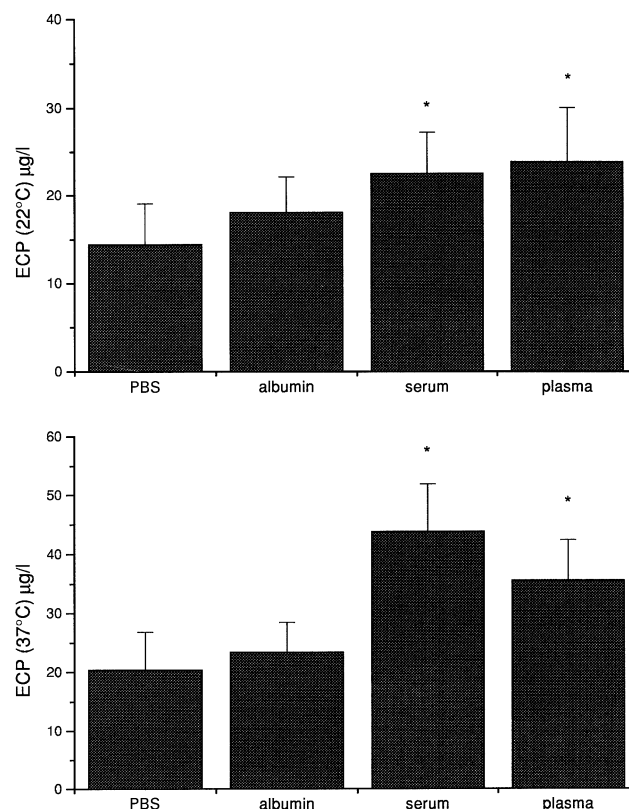


Figure 4. Purified eosinophils from healthy donors mixed with buffer, albumin solution (38 g/l), serum or citrated plasma, and Ca at 22 and 37°C. Results are presented as means and standard error of mean (SEM) of seven experiments (statistical comparison is between serum/plasma and Ca and two other groups at 22 and 37°C, respectively; results were calculated after subtraction of initial ECP values of plasmas and sera used).

temperature often is much larger. This could possibly depend on the type(s) of eosinophilic population, the activated (primed) eosinophils being more easily affected by rises in temperature. An increased propensity to release their proteins has indeed been shown with eosinophils from allergic patients during allergen exposure (18).

For some time, the coagulation process has been assumed to be an important factor for the degranulation of eosinophils. Earlier researches on the degranulation of neutrophils have shown that factor XII, prekallikrein/kallikrein, and fibronectin degradation products are important for the degranulation of neutrophils (19, 20). Thus, the same has been assumed to be valid also for eosinophils. However, our experiments with granulocytes mixed with plasma at different Ca concentrations, as well as the experiment with pure eosinophils, show that the plasma coagulation does not increase the ECP secretion. Hence, this is contrary to the earlier hypothesis that the difference in ECP values between plasma and serum is due to the plasma coagulation.

The experiments with pure eosinophils (Fig. 4) further support the conclusion that the plasma

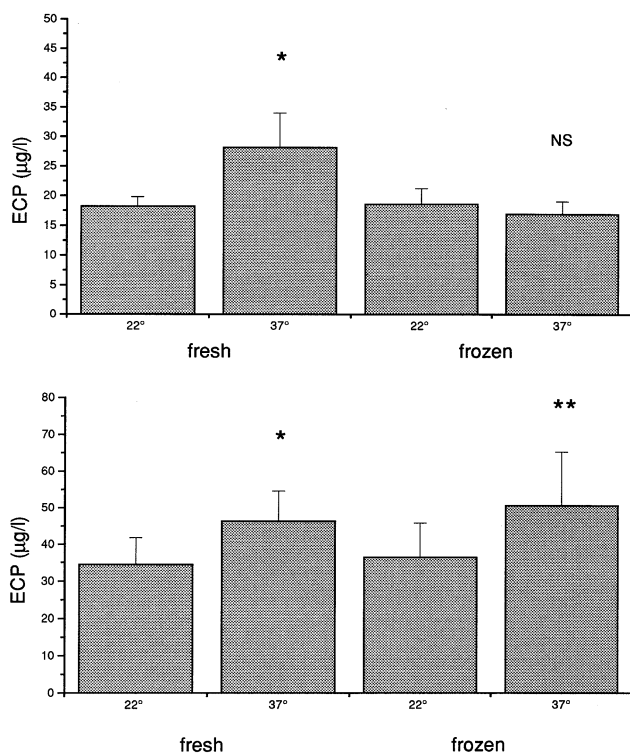


Figure 5. ECP levels after incubation of granulocytes from healthy (upper graph) and allergic (lower graph) individuals with fresh or frozen plasma, and Ca, for 1 h at 22 or 37°C. Results are presented as means and standard error of mean (SEM). $n = 13$ for healthy and $n = 10$ for allergic donors. $** = p < 0.01$; $* = p < 0.05$.

coagulation is of no importance for the ECP secretion, but that some factor in serum and plasma is of importance for the secretion of an active (temperature-dependent) kind. Hence, the protein environment is of some importance for the ECP secretion.

Our results from comparisons between granulocytes

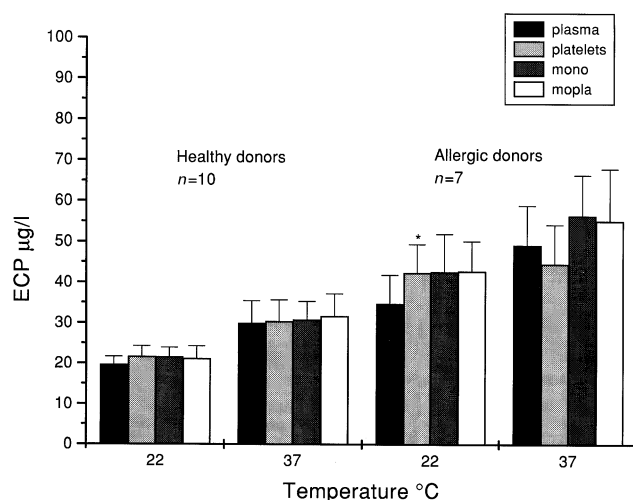


Figure 6. ECP levels after incubation of granulocytes from healthy and allergic donors with plasma and/or platelets and/or monocytes and Ca for 1 h at 22 or 37°C. mono: monocytes; mpla: monocytes and platelets. Means and standard error of mean (SEM) are shown. $n = 10$ for healthy and $n = 7$ for allergic donors.

from healthy and allergic donors when mixed with fresh or frozen plasma are consistent with other works describing two different populations of eosinophils in asthmatic and healthy individuals (21, 22). Our results also suggest the existence in plasma of freezing labile factors necessary for the secretion of ECP from healthy eosinophils, but probably not for allergic eosinophils.

Since the secretion of ECP from the granulocytes when mixed with plasma and subsequent recalcification differed from what is seen in whole blood, one might query the importance of other blood components. Our results from the experiments with platelets and monocytes do not indicate any great importance of the presence of these components for the ECP secretion.

Why, then, are ECP levels higher in serum than in plasma? The plasma coagulation in itself does not seem to be of importance, nor does the presence of platelets or monocytes. Rather, the ECP secretion from the eosinophils is partly dependent on the intrinsic activity of the cells (normal or primed), and partly on some factor (or factors) in plasma and serum. This (or these) factor(s) facilitate the secretion of ECP from normal cells, but do not seem to be necessary for allergic (primed) cells.

The reason that we do not see the same differences with temperature in our plasma-granulocyte system as in whole blood is not clear. The possibility of activation of the cells during purification may be of importance. Another important reason could be the effect of citrate.

Finally, the question arises as to how we should interpret the values of ECP in serum. It has earlier been suggested that the serum values reflect both the number of eosinophils, the degree of activation, and the amount of ECP in plasma before coagulation of the sample (12). Our results agree with this for allergic cells. However, for healthy cells, the ECP value could be affected by the above-mentioned (unknown) factor(s) in plasma, facilitating the secretion of ECP by the eosinophils.

Our results and also results from other work (13, 24) once more stress the importance of a correct handling of the blood samples before centrifugation, in order to minimize the influence of preanalytic variation, since the ECP values are affected by changes both in time and temperature before centrifugation.

Appendix: A practical procedure to correct serum levels of ECP for the influence of ambient temperature

The present study has shown that the serum levels of ECP are affected by the ambient temperature at which coagulation and sampling occur. In this appendix, we provide data that may be used to correct for the ambient temperature.

At 18–25°C, an exponential increase of approximately 15%/°C occurs (Fig. 7). Blood was taken in SST glass tubes (Becton-Dickinson, Inc.), and the increase

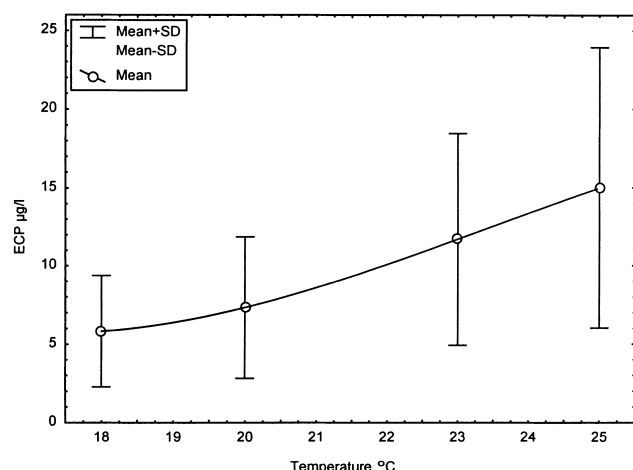


Figure 7. Influence of temperature on serum levels of ECP as illustrated by average levels of blood from 30 individuals.

was similar in blood from healthy donors ($n=10$) and from asthmatic donors ($n=20$). The temperature-dependent increase can be described by the following third-order polynomial equation:

$$[\text{ECP}] = 162.8 - 22.46X + 1.024X^2 - 0.01448X^3 \quad (1)$$

where $X = ^\circ\text{C}$. The equation is used for the calculation of the adjustment factors as shown in Table 1. The nominal temperature is set to 22°C , and the adjustment factor to 1. The adjustment factors were applied to all values (30 individuals, four temperatures) in the study, and the intracoefficient of variation before and after adjustment was calculated as shown in Table 2. The results showed that the adjustment of the ECP levels for temperature reduced the intracoefficient of variation to

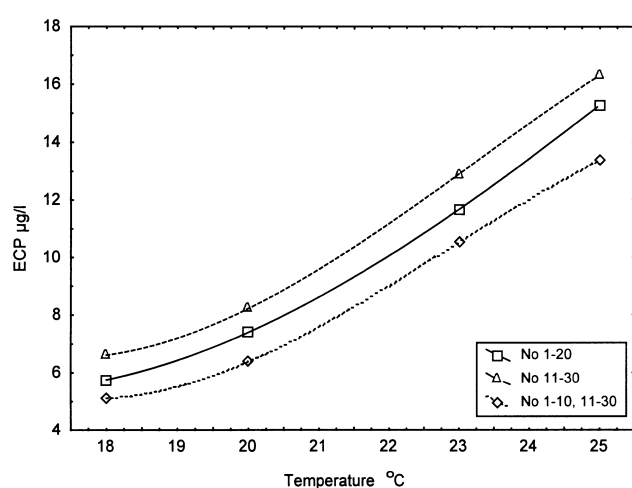


Figure 8. Influence of temperature on serum levels of ECP as illustrated by average levels of blood from 30 individuals divided into three subgroups of 20 individuals each.

Table 1. Serum levels of ECP after coagulation of blood for 1 h at different temperatures with adjustment factors given for each temperature between 18° and 25°

Temperature ($^\circ\text{C}$)	Mean ECP ($\mu\text{g/l}$)	Adjustment factor
18	5.82	1.73
19	6.37	1.58
20	7.32	1.37
21	8.58	1.17
22	10.06	1.00
23	11.68	0.86
24	13.35	0.75
25	14.98	0.67

a level that approached the coefficient of variation of the assay used, i.e., ECP CAP-FEIA (Pharmacia & Upjohn Diagnostics), and repeated blood sampling.

One bias is that adjustments were performed on the same material as used for the calculation of the adjustment factors. To control for this bias, we divided the material into subgroups of 20 individuals each (Fig. 8), after which the adjustment factors were performed. Very similar adjustment factors were obtained between the three subgroups (Table 3) and also in comparison with the whole group (Table 1). The adjustment factors, as shown in Table 4, were then used for the adjustment of the remaining 1/3 of the ECP values. This adjustment was similar to that shown in Table 2 for the whole group.

We conclude from this study that the effect of ambient temperature on the ECP levels in blood drawn in SST tubes can be reduced by the use of temperature-adjustment factors, and we recommend that this approach be adopted with the figures given in Table 1 when large variations in ECP are obtained due to variations in the ambient temperature.

Table 2. Intracoefficient of variation (CV%) before and after temperature adjustment according to Table 1

All individuals ID. 1-30	Mean ECP ($\mu\text{g/l}$)	CV % within
Before adjustment	10.0	49.9
After adjustment	10.1	13.4

Table 3. Serum levels of ECP after coagulation of blood for 1 h at different temperatures with adjustment factors given for each temperature between 18° and 25°C . Adjustment factors were calculated from average values of three subgroups with 20 individuals in each group

Adjustment factors			
Temperature ($^\circ\text{C}$)	ID 1-20	ID 11-30	ID 1-10, 21-30
18	1.74	1.69	1.76
19	1.56	1.56	1.62
20	1.36	1.36	1.41
21	1.17	1.17	1.18
22	1.00	1.00	1.00
23	0.86	0.87	0.86
24	0.75	0.76	0.75
25	0.66	0.69	0.67

Table 4. ECP and CV after adjustment

Group	Mean ECP ($\mu\text{g/l}$)	CV % within
ID 21–30	9.9	14.45
ID 1–10	7.8	14.9
ID 11–20	11.4	13.7

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