Eosinophil degranulation as an allergy activation marker
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Citation for published version (APA):
Admiraal, C. J. (2001). Eosinophil degranulation as an allergy activation marker

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Chapter 1

Introduction
**Introduction**

Eosinophils constitute a type of granulocyte and as such are derived from the bone marrow. The most distinguishing morphologic feature of the eosinophilic granulocyte is its content of distinctive cytoplasmic granules (Gleich et al. 1992). The eosinophilic granulocyte was first recognized in human blood in 1879 by Paul Ehrlich (Spry 1988). Since then, the presence of either high or low numbers of eosinophils in blood has been documented in a number of diseases (Cohen and Ottesen 1983, Weller 1984, Bruijnzeel et al. 1992, Corrigan and Kay 1992).

Involvement of eosinophils has been demonstrated especially in allergy, in asthma and in the defence against helminthic infections (Gleich and Adolphson 1986). Accumulation of eosinophils in tissues is sometimes associated with tissue damage (Gleich 1990, Holgate et al. 1991). The eosinophil expresses its biological activity by means of the products released from the cell (Gleich 1992). Eosinophil Cationic Protein (ECP) is an eosinophilic granule protein whose concentration can be established in human body fluids (Venge et al. 1977). With respect to this measurement, it has been claimed that it is indicative not only of the number of circulating eosinophils but also of their activity grade. However, there are several problems with the interpretation of this activity parameter. We have tried to obtain more insight in the activation mechanism, and we have investigated the value of this assay by detection of granule proteins in blood in relation to eosinophil count and eosinophil morphology.

**Maturation**

Eosinophils are terminally differentiated effector cells of the immune system. Eosinophilic granulocytes originate from bone marrow precursor cells. Bone marrow eosinophilic promyelocytes and myelocytes are capable of mitosis (Spry 1971). One finds a continuous flow of maturing cells via myelocyte and metamyelocyte to
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eosinophilic granulocytes; a substantial bone marrow reserve of mature cells can be mobilized on demand (Archer 1970). Spry (1971) calculated a total bone marrow transit time of 5.5 days for the eosinophil in rats. The process of post-mitotic differentiation in man takes about 2.5 days (Herion et al. 1970). Thereafter, the eosinophilic granulocyte leaves the bone marrow and moves into the blood circulation.

Little is known about the mechanisms leading to production of eosinophil progenitors from pluripotent stem cells. However, their subsequent proliferation and differentiation is influenced by humoral factors, \textit{viz.} IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Groopman et al. 1989, Ema et al. 1990, Denburg et al. 1996, Caldenhoven et al. 1998, Denburg 1998). These cytokines can induce the production of eosinophils from both human bone marrow cells and cord blood mononuclear cells in culture (Clutterbuck et al. 1989). Different eosinophilopoietins may support distinct stages of eosinophil growth and differentiation (Ueno et al. 1994, Denburg 1999).

The eosinophilic granulocyte spends 13 - 18 hours in the blood circulation before it migrates to the tissues, especially the lungs, the skin and the gut (Kroegel et al. 1994). In the vascular bed of these tissues, eosinophils are temporarily stored, creating a reservoir of cells (marginating pool) that can be rapidly recruited into the blood circulation. For each eosinophil that is present in blood there are about 300 in the bone marrow and about 100 to 300 eosinophils distributed in the tissues. Most eosinophils enter the tissue from the vascular bed and stay there for the rest of their lifetime, which is approximately 6 days (Kroegel et al. 1994). However, eosinophils that are continuously exposed to cytokines (\textit{e.g.} IL-3, IL-5, GM-CSF, TNF\textalpha) in inflamed tissue may have a lifespan of several weeks (Rothenberg et al. 1988, Clutterbuck et al. 1989). In addition to differentiation and supporting the survival time, some cytokines are also able to increase the activity grade of mature eosinophils (Weller 1992, Carlson et al. 1993) (figure 1). Therefore, it is interesting to establish whether eosinophils in individuals with higher eosinophil blood concentrations will reveal a higher activity grade.
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IL-1, IL-3, GM-CSF
Development of specific granules

IL-5, GM-CSF
Maturation

Promyelocyte

Eosinophilic myelocyte

Eosinophilic metamyelocyte

IL-5, GM-CSF

Formation of vacuoles

Activated eosinophil

Mature eosinophil

Figure 1: Eosinophil differentiation and activation

Morphology

One of the parameters that may reveal activity features of eosinophils is the morphology of these cells. A mature eosinophilic granulocyte measures 10 - 15 μm in diameter, which is similar to the neutrophil (Weller 1984). The nucleus is characteristically
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bilobed with condensed chromatin. Nucleoli are rarely present (Clark and Kaplan 1975). The most characteristic morphologic feature of the eosinophilic granulocyte is its content of distinctive cytoplasmic granules (Gleich et al. 1992). These granules are termed secondary or specific granules and stain avidly with acid dyes, such as eosin. They also have a characteristic electron-microscopic appearance with a crystallloid core (Gleich and Adolphson 1986). Besides the secondary or specific granules the eosinophilic granulocyte contains three other sorts of granule that can be detected by electron microscopy, viz. primary granules, which lack a crystallloid core and develop early in eosinophil maturation; small granules, which contain arylsulfatase and other enzymes; and microgranules, which are prominent tubulovesicular structures (Weller 1991). Eosinophils also contain organelles such as a rough endoplasmic reticulum, free ribosomes, Golgi apparatus and mitochondria (Kroegel 1994) (figure 2). Only the secondary (specific) granules are seen by light microscopy.

Eosinophils are heterogeneous with respect to morphology and functional status (Fukuda and Gleich 1989). The significance of this heterogeneity is not known. It could reflect a phase in the life cycle of the cell or an adaptation to a change in the environment. One of the aspects of heterogeneity of eosinophils is reflected in their density (specific gravity) profile (Winqvist et al. 1982, Prin et al. 1983, Prin et al. 1984, Fukuda et al. 1985, Fukuda and Gleich 1989). The eosinophilic granulocyte is the most dense leukocyte in peripheral blood. When blood specimens from patients with eosinophilia are compared with those from apparently healthy individuals, a difference in the density of the eosinophils become apparent; the eosinophils of lower density (D ≤ 1.082 g/ml) are termed ‘light density’ or ‘hypodense’ cells, whereas those of normal density (D = 1.088 g/ml) are termed ‘normodense’ cells. In patients with hypereosinophilia a large percentage of blood eosinophils is hypodense (Prin et al. 1984). Whilst normodense eosinophils represent approximately 90% of the cells found in normal individuals, in asthmatic patients the mean percentage of hypodense blood eosinophils varies between 35 and 65% (Fukuda et al. 1985, Shult et al. 1988, Frick et al. 1989). Experiments
suggest that reduction of density may be an early sign of eosinophil activation and incipient degranulation (Winquist et al. 1982, Prin et al. 1983, Prin et al. 1984, Fukuda et al. 1985). In patients with bronchial asthma, the numbers of hypodense eosinophils correlate positively with the degree of blood eosinophilia (Fukuda et al. 1985). Heterogeneity of eosinophils is also reflected in morphology. Eosinophils are usually circular or ovoid when observed by light microscopy, but cells with one or more pseudopods have also been observed in human peripheral blood, sputum, bone marrow and nasal smears (Hanker et al. 1981). These cells have been named ‘medusa cells’, to emphasize their length and the presence of pseudopods. The significance of ‘medusa cells’ is unknown. Electron microscopic studies of tissue eosinophils in human biopsies from patients with a wide variety of diseases show numerous differences in their morphology when compared to circulating eosinophils obtained from healthy donors (Dvorak 1994). Activated tissue eosinophils may contain increased numbers of lipid bodies, primary and small granules and tubulovesicles. Lipid bodies may also increase considerably in size and display focal lucencies (Dvorak et al. 1991). In tissue eosinophils the number of specific granules with an empty appearance (e.g. vacuoles) increases (Dvorak and Ishizaka 1994). The presence of cytoplasmic vacuoles and loss of the dense core of specific granules can be associated with secretion of granule contents. This means that these tissue eosinophils may be degranulated eosinophils (Dvorak et al. 1991).

There are also studies in which the morphology of blood eosinophils has been investigated in more detail (Tai and Spry 1976, Spry 1981, White 1986). It cannot be assumed that activated blood eosinophils have identical properties to tissue eosinophils, because blood eosinophils have not met analogous stimuli that affect eosinophils at tissue sites. However, many alterations are comparable to those observed with tissue eosinophils. In acquired disorders, eosinophils may be vacuolated or agranular, such as demonstrated in reactive eosinophilia with parasitic infections (Tai and Spry 1976, Spry 1981, White et al. 1986). Bain (1989) mentions that the most characteristic change in the
hypereosinophil syndrome is a slight increase in eosinophil nuclear lobulation or the presence of ring-shaped nuclei. The significance of segmentation of granulocyte nuclei is not known, but the hypothesis that the number of nuclear lobes reflects the age of the cell is usually accepted (Arneth 1920). However, it is also possible that this phenomenon reflects an activity grade (Tai and Spry 1976). Several studies have been performed by electron microscopy and others by light microscopy. Light microscopy might be an easy way to determine differences in the morphology of eosinophilic granulocytes. Besides studying eosinophils in patients with eosinophilia, the morphologic characteristics of eosinophils under normal conditions should also be described. We have studied alterations in the morphology of eosinophilic granulocytes in relation to their concentration in blood and their activity grade as measured by the property to release granule proteins.

Figure 2: Morphology and characteristic organelles of the mature eosinophil granulocyte.
Eosinophil functions

Eosinophils have several effector functions. It is well established that a blood and tissue eosinophilia is associated with helminthic infections and that eosinophils are also a feature of allergic inflammation (Gleich and Adolphson 1986). For executing their effector functions, the cells should migrate to the site of inflammation. This transfer is a multistep process, consisting of first rolling and adhesion to vascular endothelium, followed by transmigration through the endothelium (diapedesis) and chemotaxis to the specific site (Rothenberg 1998). During this process eosinophils are primed or preactivated. The term ‘primed’ refers to a state in which the cell is not yet activated. The primed cell does not respond autonomously but reacts more rapidly and to a greater extent to a subsequent activating stimulus (Henson et al. 1992). When the primed cell enters the end organ, it can be stimulated by a combined effect of several mediators. The ultimate effector function, e.g. the killing process, is mediated by the production of reactive oxygen metabolites (respiratory burst) and the release of cytotoxic proteins (degranulation). The respiratory burst results in the generation of toxic oxygen metabolites, including superoxide anions and hydrogen peroxide, which together with the protein Eosinophil Peroxidase (EPO), generate the highly tissue-damaging hypohalous acids in the immediate environment of the cell (Gleich and Adolphson 1986, Gleich et al. 1992). Degranulation results in the release of cationic proteins, such as Eosinophil Cationic Protein (ECP) or Eosinophil Protein X (EPX), which have cytotoxic activities (Gleich and Adolphson 1986, Gleich et al. 1992).

Granule proteins

An eosinophilic granulocyte contains several species of granule, as described above. Granules differ not only in appearance but also in content.
Microgranules are considered to be small parts of the reticulo-endothelial system. They contain albumin (Berger et al. 1991). Small granules (< 0.5 μm) have been shown to contain hydrolytic enzymes (acid phosphatase, arylsulfatase) and catalase (Parmley and Spicer 1974). Primary granules are round and uniformly electron-dense, and they are characteristically seen in eosinophilic promyelocytes. In the primary granules, only the Charcot-Leyden Crystal (CLC) protein has been determined (Gleich et al. 1992). In addition, eosinophils also contain non-membrane bound lipid-rich inclusions, called lipid bodies (Gleich and Adolphson 1986, Venge 1990).

In this study, we focussed on proteins of the secondary (specific) granules. Four distinct proteins, which comprise about 90% of the granule proteins, have been isolated and characterized (Egesten et al. 1986). These proteins are known as Eosinophil Cationic Protein (ECP), Eosinophil Protein X (EPX), Eosinophil Peroxidase (EPO) and Major Basic Protein (MBP) (Venge 1990) (table I). The crystalloid, electron-dense core in secondary granules is composed of MBP. In the matrix compartment ECP, EPX and EPO are present (Egesten et al. 1986). The common characteristics of these four proteins are their high isoelectric points, ranging from about pH 9 to pH 11 (Gleich and Adolphson 1986). Another feature is their cytotoxic activity. Thus, all four have been shown to be able to kill both mammalian and non-mammalian cells (Gleich et al. 1979, McLaren et al. 1981).

Eosinophil Cationic Protein is a heterogeneous protein with respect to molecular mass, which ranges from about 18-21 kD (Abu-Ghazaleh et al. 1992, Venge and Byström 1998, Venge et al. 1999). This heterogeneity is caused by differences in glycosylation of the protein. Upon release, deglycosylation of ECP occurs (Rosenberg and Tiffani 1994). Glycosylation may protect cells from toxic ECP effects during storage, and deglycosylation may therefore be a way of increasing the cytotoxic properties of ECP. Its cytotoxic activity may be due to the fact that ECP is capable of making channels in
lipid membranes that allow the passage of water and other small molecules (Young et al. 1986). In most systems ECP is by far the most cytotoxic of the four proteins. The amino-acid sequence shows 66% homology to EPX and 31% homology to pancreatic ribonuclease (Gleich and Adolphson 1986). Thus, ECP belongs to a ribonuclease multigene family. ECP causes direct histamine release from human basophils and was found to inhibit lymphocyte proliferation in vitro (Peterson et al. 1986). In addition, ECP has been shown to enhance the coagulation activity of plasma through a mechanism dependent on activation of coagulation factor XII and also to enhance the plasminogen-activating activity of urokinase (Venge et al. 1979, Dahl and Venge 1979).

Eosinophil Protein X is a protein with a molecular mass of about 18 kD. EPX and eosinophil-derived neurotoxin (EDN) are considered to be identical (Peterson and Venge 1983). EDN was initially so called because of its capacity to induce cerebellar dysfunction when injected intracerebrally in hamsters, i.e. the Gordon phenomenon (Fredens et al. 1982). EPX/EDN is the least basic of the four eosinophil granule proteins but still has an isoelectric point of about 9 (Gleich et al. 1992). EPX shows a 40% amino-acid sequence homology with ECP in the N-terminal part of the molecule. EPX has been found to be a very active ribonuclease (Gleich and Adolphson 1986). The cytotoxicity of EPX against parasites is less than that of MBP or ECP (Gleich et al. 1992).

Eosinophil peroxidase is a heterodimeric peroxidase with a light chain of 15 kD and a heavy chain of 52 kD. It is 60% homologous with myeloperoxidase of neutrophils (Sakamaki et al. 1989). EPO forms a potent cytotoxic principle together with a halide and H₂O₂ (Jong et al. 1981). In addition, EPO triggers degranulation of mast cells (Henderson et al. 1980).

Major Basic Protein has a molecular mass of 13.9 kD and a calculated pI of 10.9. Within the secondary (specific) granules a unique structure exists, a crystalloid core, which can only be seen by electron microscopy (Gleich and Adolphson 1986). MBP has a high tendency to polymerize and is stored in the granules as typical crystalloid formations.
MBP probably acts by crosslinking membrane structures (Gleich et al. 1992). It is not entirely specific for eosinophils since it is also found in low amounts in basophils and in considerable amounts in some placental cells (Gleich et al. 1992).

The total amount of granule proteins in eosinophils may vary (Abu-Ghazaleh et al. 1992, Kita et al. 1992, Kaneko et al. 1995) (table I). Winqvist et al. (1982) and Carlson et al. (1994) have shown that eosinophils with higher density contain more ECP per eosinophil. This observation can possibly be explained by the fact that activated eosinophils show a decreased quantity of specific granules (Dvorak 1991). The method of extraction, used to determine the total amount of granule proteins can also yield different results (Carlson 1994).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol mass</th>
<th>pI</th>
<th>site</th>
<th>total amount (pg/eo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECP</td>
<td>18-21 kD</td>
<td>10.8</td>
<td>matrix</td>
<td>5.3*, 4.0**, 13.5****</td>
</tr>
<tr>
<td>EPX</td>
<td>18-19 kD</td>
<td>8.9</td>
<td>matrix</td>
<td>3.3*, 2.3***, 9.1****</td>
</tr>
<tr>
<td>EPO</td>
<td>15+52 kD</td>
<td>10.8</td>
<td>matrix</td>
<td>12.2*</td>
</tr>
<tr>
<td>MBP</td>
<td>14 kD</td>
<td>10.9</td>
<td>core</td>
<td>8.9*</td>
</tr>
</tbody>
</table>

* Abu-Ghazaleh et al. 1992
** Kita et al. 1992
*** Kaneko et al. 1995
**** Carlson et al. 1994
Degranulation

Activated eosinophilic granulocytes are involved in inflammation and allergy by release of granule proteins. Cytotoxic proteins of eosinophils are stored in cytoplasmic granules, which function as a storage compartment until the content is released to the outside of the cell or into phagosomes. The mechanism of degranulation by which ECP and other cationic granular proteins are released from eosinophils is poorly understood. Regulation of the secretion of eosinophilic granule proteins is complex, as shown by the wide range of responses of eosinophils to different stimuli (Weller 1991). Eosinophils possess both high (FcεRI) and low (FcεRII) affinity receptors for IgE, which may permit them to participate directly in the response to allergens (Weller 1991). Also surface-bound complement, immunoglobulins and soluble secretagogues may provide stimuli for granule release. Serum-opsonized non-phagocytosable beads are potent inducers of granule release, mainly depending on their content of C3-derivatives on their surface (Winqvist et al. 1984). IgG and (s)IgA receptors on eosinophils may stimulate selective release of granule proteins (Abu Ghazaleh et al. 1989). These stimuli induce degranulation when eosinophils are attached to a surface (Horie and Kita 1994).

Eosinophil degranulation on Ig-coated or C3b-coated beads is potentiated in experiments in vitro by preincubation (priming) with GM-CSF, IL-3 and IL-5 (Fujizawa et al. 1990, Carlson et al. 1993). Soluble secretagogues include GM-CSF, PMA and platelet-activating factor (PAF). PAF is an activator of eosinophils, which is synthesized and released by stimulated eosinophils. Cytochalasin B, a priming agent of eosinophil degranulation, disrupts the cytoskeleton of the cells, but the exact mechanism of its enhancing effect on degranulation is not known. The calcium ionophore A23187 causes calcium-dependent degranulation in eosinophils (Fukuda et al. 1985).

Apart from their biological activities, ECP or EPX levels in biological fluids may be used as markers of eosinophil degranulation. To investigate the phenomenon of degranulation of eosinophils and to study the activation mechanisms in health and disease, immunoassays for ECP and EPX have been developed (Venge et al. 1977).
Eosinophils in pathological conditions

Initially, the eosinophilic granulocyte was considered to have an exclusively protective role, for example in host defence against parasites (Dessein and David 1982). Later, the eosinophil was recognized as a pro-inflammatory cell that mediates allergic symptoms (Gleich and Adolphson 1986).

Eosinophils normally constitute only a small proportion of the circulating leukocytes, with a reference range from 0 to $0.5 \times 10^9/l$. A diurnal variation occurs, with eosinophil blood counts peaking at night and being at the lowest level in the morning. Increased amounts of eosinophils may be present in blood and inflammatory tissues of individuals with allergic diseases such as asthma (Buijnzeel et al. 1992, Smith 1992, Barnes 1996), allergic rhinitis (Mullarkey et al. 1980), hypereosinophilic syndrome (Fauci et al. 1982), neoplasmatas (Slungaard et al. 1983) and (auto) immune diseases (ulcerative colitis) (Makiyama et al. 1995). In a study with 43 asthmatic individuals, the number of eosinophils in affected tissues correlated with the severity of the clinical symptoms (Bousquet et al. 1990).

Another marker of eosinophils is the measurement of secretory granule proteins (e.g. ECP) in biological fluids. With application of a commercially available kit, the levels of ECP can be evaluated. In various diseases, the concentrations of eosinophilic granule proteins in serum and tissue fluids, such as bronchoalveolar lavage, sputum, intestinal fluid and urine, have been established (Durham et al. 1989, Bousquet et al. 1993, Kristjansson et al. 1996). In many of these materials, the levels of these eosinophilic proteins are higher than what is necessary to cause in vitro injury of the cells (Venge et al. 1988). Therefore, it is obvious that the eosinophilic granulocyte is actively involved in many inflammatory diseases.

In several studies, laboratory parameters have been correlated with clinical and lung function parameters (Bonini et al. 1993, Ferguson et al. 1995). In asthmatic patients, lung function and bronchial hyperreactivity in relation to serum ECP have been studied.
(Zimmerman et al. 1993, Wever et al. 1994, Roquet et al. 1996, Skedinger et al. 1996). It has been concluded that serum ECP concentrations might be a clinically useful tool for assessing the severity of bronchial symptoms (Roquet et al. 1996, Skedinger et al. 1996). However, in other studies, serum ECP has been demonstrated to be only a poor indicator of disease activity in individuals with chronic asthma (Ferguson et al. 1995, Hoekstra et al. 1998). Thus, the validity of serum ECP concentrations as a marker of, for example, asthma activity may be questioned (Niimi et al. 1999).

A few studies have described the relationship between serum ECP and clinical or functional scores in patients with ulcerative colitis (Makiyama et al. 1995, Luck et al. 1997). In the active state of the disease, the colonic mucosa exhibit infiltration sites with numerous eosinophils. The eosinophils were classified as active cells, positively stained by an antibody to the secreted form of ECP (EG2). Makiyama et al. (1995) also showed higher concentrations of serum ECP in patients with active ulcerative colitis compared with normal individuals and patients with inactive ulcerative colitis. However, the additional value of serum ECP in comparison with eosinophil numbers in blood has not been demonstrated.

For monitoring of seasonal respiratory allergy in patients treated with specific immunotherapy, measurement of eosinophils and ECP can be a useful diagnostic tool (Rak 1993, D'Amato et al. 1996). Immunotherapy is a well-documented method of treatment for allergic rhinitis, especially in pollen-induced disease. Initially, such studies were focused on number, differentiation and activation of lymphocytes and the switch between Th1 and Th2 cells. Recent developments in the field of cytokines resulted in a wider view. Th2 lymphocytes were found to produce cytokines responsible for differentiation, activation and viability of eosinophils (Moens et al. 1994).

Because glucocorticoids are potent anti-inflammatory drugs, the effect of their administration on eosinophilic inflammation has been studied (Pedersen et al. 1993,
Zimmerman and Tsui 1993, Selroos et al. 1994). A broad range of diseases in which eosinophils play an important role, as mentioned above, are effectively treated with glucocorticoids. Despite the extensive use of inhaled or orally supplied glucocorticoids in the treatment of asthma, ulcerative colitis and immediate hypersensitivity allergic conditions, the mechanism is poorly understood. Actions of corticosteroids are mediated through binding to a DNA-binding protein. This interaction leads to target gene activation or suppression. Activation or suppression occurs in cells that are involved in the pathophysiology of inflammation and causes an increase or decrease, respectively, in the production of mediators (Pederson and O'Byrne 1997). It is known that glucocorticoids may result in eosinophilopenia (Morris 1985). The affected mediators may negatively influence eosinophil differentiation or eosinophil migration to inflammatory tissues (Fauci et al. 1976). Nittoh et al. (1998) revealed that glucocorticoids inhibit the survival of rat peritoneal eosinophils by enhancing eosinophil apoptosis.

Other mediators may result in effects on cellular functions and influence the activity grade of eosinophils, for instance the propensity to release granule proteins (Fauci et al. 1976). Therefore, it is interesting to study the effect of glucocorticoids on blood eosinophil numbers and serum ECP concentrations. In clinical studies, we have investigated the possibility to distinguish patients from apparently healthy controls by measuring these parameters. Subsequently, we have determined the influence of corticosteroid treatment on these laboratory parameters.

Measurement of eosinophil degranulation

As described previously, ECP or EPX levels in biological fluids may be used as markers of eosinophil degranulation. There is no additive value in measuring both proteins simultaneously (Reimert et al. 1993, Pedersen et al. 1993, Robinson et al. 1995, Rao et al. 1996). High correlations between ECP and EPX levels have been established.
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(Reimert et al. 1993, Rao et al. 1996). ECP is considered to be a specific indicator of eosinophil activity, because the content in other cells is negligible. EPX is also present in neutrophils, albeit to a very small extent, i.e. 1 - 2% of the content of an eosinophil. Therefore, we have decided to measure ECP. However, several problems should be considered when measuring cell-derived products. For measuring the degree of eosinophil activation, anticoagulated plasma cannot be used, because the concentration of ECP or EPX in plasma is very low (Reimert et al. 1991, Pena et al. 1996, Rubira et al. 1997, Venge et al. 1999). Granule proteins released in vivo will adhere to tissues; e.g. positively charged and sticky proteins may be expected to bind to tissue elements before reaching the blood compartment. In serum, much higher concentrations of ECP are found. Thus, ECP is released during in vitro clotting. One hypothesis is that allergen exposure to inflammatory tissues gives rise to cytokine formation, which activates eosinophils in vivo locally and pre-activates eosinophils systemically (Venge 1993). During clotting in vitro, these in vivo primed eosinophils will become activated, and proteins are released in the serum. In healthy individuals, eosinophils are in majority not pre-activated, which means that they will release lower quantities of granule proteins during in vitro clotting (Venge 1993). In this hypothesis, the extent to which eosinophils will release ECP during in vitro clotting may reflect the state of pre-activation of the eosinophil population in vivo. The higher the ECP levels in serum, the higher may be the propensity of the eosinophils to release their content when attracted to the site of inflammation. However, serum ECP levels do not necessarily reflect in vivo release of granule proteins, because activation during in vitro clotting can result in different signals to the cells than those generated during in vivo inflammation. It is not known which agents, liberated during in vitro clotting, are responsible for degranulation of eosinophils (Björk et al. 2000). During clotting, PAF released from platelets may activate eosinophils to degranulate, or complement activation is induced. C3b coupled to a surface is able to enhance degranulation of eosinophil (Carlson et al. 1993). Due to variable clotting conditions, the different ECP results may lead to problems in
clinical interpretation (Reimert et al. 1993). For clinical interpretation, it is a drawback that many publications on eosinophil-derived proteins in serum do not provide any particular information with respect to preanalytical conditions (Juntunen-Backman et al. 1993, Roquet et al. 1996). Blood sample processing methods will affect serum ECP concentration. Factors like incubation time and temperature during sample processing are of significant importance for appropriate clinical interpretation of the results (Reimert et al. 1993, Kurihara et al. 1992). Clotting for 2 hours at 37°C has been demonstrated to result in 2 - 3 times higher serum ECP concentrations compared with clotting for 2 hours at 21°C (Reimert et al. 1993). Another study showed a difference of 20 times between clotting at 37°C and 0°C (Pena et al. 1996). Increased serum ECP concentrations may be due to priming of eosinophils for increased degranulation by contact with the equipment used for blood sampling and not by conditions encountered in vivo (Rubira et al. 1997). Therefore, the kind of blood sampling tube and equipment should be specified for correct interpretation of the results (Reimert et al. 1993, Rubira et al. 1997).

In several studies, a linear correlation between serum ECP and eosinophil count has been observed (Griffin et al. 1991, Sont et al. 1993, Björnsson et al. 1994). Serum ECP perhaps only reflects the number of eosinophils present in the blood sample, if clotting activates the complete eosinophil population instead of only the preactivated eosinophils. Therefore, measuring serum ECP under standardized preanalytical conditions may reflect a combination of the number of eosinophils and their state of activation. In clinical studies, the additional value of serum ECP over eosinophil counts can be investigated. For determination of the activity grade of the eosinophils it is recommended to calculate the ECP release per eosinophil. In addition, it should be emphasized that the intracellular ECP content of eosinophils is about 50 - 1000 times as high as the ECP concentration in serum (Carlson et al. 1991). Thus, the amount of ECP actually released during in vitro clotting is only a minor fraction of the total content of ECP in the eosinophils. In vitro clotting does not result in such a strong activation that
eosinophils release their total content of granule proteins. In most studies, the ECP released per eosinophil has not been calculated. Moreover, the serum ECP is highly correlated with eosinophil counts, so the additional value of serum ECP levels over blood eosinophil counts is not obvious (Björnsson et al. 1994, Skedinger et al. 1995). In our study with several patient groups, eosinophils, ECP in serum and ECP released per eosinophil (ECP/eosinophil ratio) have been investigated in relation to the clinical state, and serum ECP has been measured under standardized preanalytical conditions.

**Aim of the study**

Although many studies have contributed to our understanding of the specific role of eosinophils and the clinical effects of eosinophil degranulation, several questions remain to be answered.

Items that emerge are:

1. Pre-analytical factors and analytical circumstances that influence serum ECP concentrations and the interpretation of this parameter.

2. The additional value of serum ECP with respect to blood eosinophil concentrations in monitoring disease activity and efficacy of treatment, as well as distinguishing diseased individuals from apparently healthy controls.

3. Investigation of the clinical significance of other factors that can be used as markers of eosinophil activation, *e.g.*
   - morphology of eosinophils
   - ECP released per eosinophil
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