Eosinophils Maintain Their Capacity to Signal and Release Eosinophil Cationic Protein Upon Repetitive Stimulation with the Same Agonist

Hans-Uwe Simon, Martina Weber, Eva Becker, Yael Zilberman, Kurt Blaser and Francesca Levi-Schaffer

J Immunol 2000; 165:4069-4075; doi: 10.4049/jimmunol.165.7.4069
http://www.jimmunol.org/content/165/7/4069
Eosinophils Maintain Their Capacity to Signal and Release Eosinophil Cationic Protein Upon Repetitive Stimulation with the Same Agonist


Eosinophils contain in their granules eosinophil cationic protein (ECP) and other basic proteins that have been implicated in immunity to parasites and pathophysiology of chronic allergic responses. In a model of eosinophil degradation, we show that eosinophils release ECP upon short-term GM-CSF priming and stimulation with either platelet-activating factor (PAF) or the anaphylatoxin C5a, but not eotaxin. Restimulation with the same agonist (PAF or C5a) was unsuccessful as assessed by monitoring intracellular calcium concentration and ECP release. In contrast, upon an intermediate washing step, eosinophils rapidly transduced PAF and C5a signals followed by significant ECP releases. Ligand-binding studies demonstrated that only a proportion of PAF receptors is internalized upon cell stimulation and that washing of the cells removes the agonist from the cell surface. Upon repetitive stimulation, eosinophils with less than 50% of the original ECP content were obtained. Such eosinophils did not increase cellular ECP levels even in the presence of the eosinophil survival factor GM-CSF in overnight cultures. In vivo studies revealed that eosinophils always express detectable amounts of ECP under chronic inflammatory conditions. In conclusion, we have shown that eosinophils maintain their capacity to degranulate upon repetitive stimulation with the same agonist as long as the receptor is not occupied from a previous stimulation. The cellular content of ECP appears to be a no limiting factor in the case of repetitive stimulation, implying that mature eosinophils may not require a significant ECP resynthesis. The Journal of Immunology, 2000, 165: 4069 – 4075.

Inflammatory disorders are characterized by an expansion of hemopoietic effector cells. In allergic and parasitic diseases, the cellular infiltrate consists mainly of eosinophils. Several mechanisms are involved in this process, such as increased eosinophil production in the bone marrow, preferential recruitment, and chemotaxis to the site of inflammation, as well as delayed apoptosis (1). At the site of inflammation, eosinophils release toxic cationic proteins upon stimulation, a process thought to be important in host defense (2). Tissue damage caused by eosinophil granule proteins may also be important in the pathophysiology of asthma, atopic dermatitis, and other chronic allergic diseases.

There have been a number of studies describing eosinophil activation mechanisms. Hematopoietins, such as IL-3, IL-5, and GM-CSF (4), increase functional responses of eosinophils to various agonists, including lipid mediators, complement factors, or chemokines (3–6). This effect of hematopoietins, called “priming,” is also observed in other granulocyte subtypes (7).

Priming of eosinophils appears to be required for ligand-induced degranulation (8).

Most of the activation studies have focused on the response of eosinophils to a single step of activation. However, because the eosinophils may live in the inflamed tissue for more than a week (9), it is likely that the same ligand stimulates the cell repeatedly or continuously. Therefore, we have studied the effect of repetitive stimulation with the same agonist in an in vitro model of eosinophil activation. We demonstrate that GM-CSF-primed eosinophils can be activated by platelet-activating factor (PAF) or complement factor C5a to release eosinophil cationic protein (ECP) up to six times. Moreover, it was found that one major mechanism of temporary eosinophil unresponsiveness by agonist-induced stimulation appears to be receptor inactivation by the agonist itself.

Materials and Methods

Subjects

A group of 15 atopic dermatitis patients, 2 patients with the hypereosinophilic syndrome, and 4 healthy control individuals were studied. All patients with atopic dermatitis fulfilled the diagnostic criteria of Hanifin and Rajka (10). The criteria for the diagnosis of hypereosinophilic syndrome were: at least 1500 eosinophils per μm³ blood for longer than 6 mo, the absence of evidence of parasitic infections, allergic diseases, or other disorders associated with eosinophilia, and the infiltration of tissues by eosinophils (11). At the time of the study, neither patients nor control individuals received systemic corticosteroid treatment. Heparin anticoagulated blood (50 ml) was collected under standard hospital-approved protocols for immunologic monitoring. Informed consent was obtained from all patients and control individuals, and the study was approved by the Swiss Academy of Medical Science represented by the Medical Ethics Committee of Davos.

Abbreviations used in this paper: PAF, platelet-activating factor; ECP, eosinophil cationic protein.

1 Swiss Institute of Allergy and Asthma Research, University of Zurich, 2 Clinic for Dermatology and Allergy (Alexanderhausklinik), Davos, Switzerland; and 3 Department of Pharmacology, School of Pharmacy, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Received for publication April 17, 2000. Accepted for publication July 18, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by grants from the Aimwell Charitable Trust (U.K.), the Swiss National Science Foundation (Grants 32-49210.96 and 31-58916.99), the Sauer Foundation Zurich, and Pharmacia & Upjohn, Dubendorf (Switzerland).

Current address: Department of Pharmacology, University of Bern, CH-3010 Bern, Switzerland.

Address correspondence and reprint requests to Dr. Francesca Levi-Schaffer, Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91120, Israel. E-mail address: fls@cc.huji.ac.il

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00

0022-1767/00/S02.00
Media and reagents

Complete culture medium was RPMI 1640 (Life Technologies, Basel, Switzerland) supplemented with 2 mM L-glutamine, 200 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS (all from Life Technologies). Buffer A was in mM: NaCl 140, KCl 1, MgCl2 1, glucose 10, CaCl2 1, and HEPES 20, pH 7.23 (Sigma, Buchs, Switzerland). Fura-2/AM and ionomycin were from Boehringer Mannheim (Roche, Switzerland). GM-CSF was a kind gift from Dr. T. Hartung (University of Konstanz, Konstanz, Germany). PAF and lyso-PAF were from Calbiochem (Lucerne, Switzerland). Fluorescent PAF and lyso-PAF (BODIPY fluorophore-conjugated) were purchased from Molecular Probes (Eugene, OR). The specific PAF receptor antagonist WEB 2086 was a kind gift from Dr. C. Meade (Boehringer Ingelheim, Ingelheim, Germany). CsA was from Sigma and retinoic acid from PeproTech (distributed by Juro Supply AG, Lucerne, Switzerland). Anti-CD16 mAb microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-ECP mAb (clone EGI) was obtained from Pharmacia Diagnostics (Uppsala, Sweden). Unless stated otherwise, all other reagents were from Sigma.

Eosinophil purification

Human eosinophils were purified as previously described (12–16). Briefly, PBMC were separated from peripheral blood by centrifugation on Ficoll-Hypaque (Snedden-Fakola, Bisch, Switzerland). The lower phase, mainly granulocytes and erythrocytes, was treated with erythrocyte lysis solution (155 mmol/L NH4Cl, 10 mmol/L KHCO3, and 0.1 mmol/L EDTA, pH 7.3). The resulting cell populations contained mainly granulocytes. To purify eosinophils, the granulocyte population was incubated with anti-CD16 mAb microbeads. CD16+ neutrophils were depleted by passing the granulocyte cell separation system (Miltenyi Biotec) with column type C and an attached 21-gauge needle in the field of a permanent magnet. The resulting cell populations contained 99% eosinophils as determined by staining with Diff-Quik (Baxter, Dodingen, Switzerland) and light microscopy.

Eosinophil cultures

Eosinophils were cultured at 1 × 10^6/ml in the presence or absence of GM-CSF, PAF, CsA, or eotaxin for the indicated times using complete culture medium and stored in the dark until analysis at 37°C. Cells were washed and resuspended in buffer A immediately before use. Cells were continuously monitored and stirred in 1.9 ml buffer A at 37°C in a quartz cuvette (Hellma, Basel, Switzerland) in a FluoroMax spectrophotometer (Spex Industries, Edison, NJ) and analyzed with the DM3000 Cation Measurement software (Spex Industries). Each analysis was calibrated by addition of 1 μM ionomycin and 0.02% Triton X-100 followed by 15 mM EGTA. Changes in cytosolic free calcium were calculated as the peak value obtained within the first minute of agonist stimulation minus the baseline value measured before stimulation.

ECP measurements in blood eosinophils

ECP levels were measured in eosinophil lysates and supernatants using the Pharmacia UniCAP System for ECP (Pharmacia & Upjohn, Dubendorf, Switzerland) according to the manufacturer’s instructions. The detection limit was 2 μg/L. Samples with more than 200 μg/L were diluted and remeasured. Total ECP content was determined in eosinophil lysates, which were obtained by treating eosinophils (1 × 10^6/ml) with 0.2% Triton X-100. Supernatants of stimulated eosinophils containing released ECP were also tested.

ECP measurements in tissue eosinophils

ECP expression in eosinophils was determined in several eosinophilic tissue biopsies by immunohistochemistry as previously described (9). The following tissues were investigated: 1) nasal mucosa from a patient with allergic rhinitis; 2) skin from a patient with atopic dermatitis; 3) bladder from a patient with cancer; 4) bone with eosinophilic granuloma; 5) stomach from a patient with eosinophilic gastroenteritis; and 6) intestine from a patient with eosinophilic gastroenteritis. Immunostaining was performed with anti-ECP mAb using the alkaline phosphatase-anti-alkaline phosphatase method with a commercial kit (Dako, Glostrup, Denmark) according to the manufacturer’s instructions.

ECP mRNA measurements

mRNA expression of ECP was studied using RT-PCR (9, 13, 17). Primers for ECP (5'-CAG TCT GAA CCC CTC-3' and 5'-CCG TGG AGA ATC CCG TG-3') were designed based on the published human ECP sequence (18) and synthesized by Microsynth (Balgrach, Switzerland). For negative controls, PCR were performed without template DNA. Control amplifications were performed using primers for G3PDH (17). The amplification products (ECP, 315 bp; G3PDH, 190 bp) were separated on 1.5% agarose gels and visualized by ethidium bromide staining. In some experiments, PCR products were transferred to a nitrocellulose filter, which were hybridized with fluorescein-12-dUTP ECP probe (DuPont NEN Research Products, Boston, MA). The ECP cDNA used for the probe was cloned by PCR amplification of human eosinophils, and its specificity confirmed by sequencing. A specific HRP-conjugated Ab was used to detect fluorescein-labeled DNA. The blots were developed by an enhanced chemiluminescence technique according to the manufacturer’s instructions (DuPont NEN).

Statistical analysis

Statistical analysis was performed by using Student’s t test. A p value of <0.05 was considered statistically significant.

Results

ECP levels do not differ between blood eosinophils derived from normal control individuals and eosinophilic patients

As shown in Fig. 1A, ECP mRNA levels varied between eosinophil populations derived from different individuals. However, the
levels in four patients with atopic dermatitis appeared to be similar to those observed in four healthy controls. Similar data were seen when total ECP contents were compared (Fig. 1B). ECP expression did not differ between control individuals and patients with atopic dermatitis. In addition, purified eosinophils from two patients with the hypereosinophilic syndrome had similar cellular ECP levels.

**PAF and C5a but not eotaxin release ECP from GM-CSF-primed eosinophils**

We next searched for a system for ECP release from peripheral blood eosinophils using physiologic agonists. As shown in Fig. 2, significant ECP release was observed when eosinophils were pre-treated with GM-CSF and subsequently stimulated with optimal concentrations of PAF or C5a. If these three agonists were used alone, no significant release of ECP was observed. Interestingly, activation with PAF and subsequent stimulation with GM-CSF was not associated with an increased ECP release. In contrast to PAF and C5a, eotaxin did not induce a significant ECP release from GM-CSF-primed eosinophils. In preliminary experiments, we established the optimal time for GM-CSF priming (20 min) and subsequent PAF or C5a stimulation (both 25 min). Ten-minute and 40-min incubations for priming or degranulation stimulation were clearly less effective (not shown).

**Eosinophils release ECP upon a second stimulation with the same agonist**

Stimulation of cells by agonists is usually followed by a time period of unresponsiveness, also called “desensitization.” In this time period, cells do not show a functional response upon stimulation either with the same or another ligand, which binds to the same (19, 20) or different receptors with same signal transductions pathways (21). However, as shown in Fig. 3, eosinophils could be triggered to a second ECP release by the same agonist when the cells were washed after the first stimulation. Already 5 min after the first stimulation, eosinophils responded to either PAF or C5a activation. The response to the second PAF stimulation after 20 min was as high as the first response. The second C5a response reached its maximum after 45 min, but was always less in comparison to the ECP levels released upon the first stimulation. We stimulated GM-CSF-primed eosinophils with PAF up to six times within 5 h and always observed a significant release of ECP (Table I). After six stimulations, the eosinophils still contained more than 50% of the original ECP content (Table I, mean ± SEM of total cellular ECP levels of experiments 2, 5, and 6: unstimulated cells 1762 ± 44 µg/L, after six stimulations 1007 ± 110 µg/L).

**A second stimulation with the same agonist increases cytosolic free calcium**

We next investigated agonist-induced changes in intracellular calcium levels to evaluate receptor-mediated signaling mechanisms. Both PAF and C5a led to rapid, transient, and dose-dependent changes in intracellular free calcium concentrations (not shown). Peak calcium levels were observed within 1 min of addition of 10⁻⁷ M PAF or 10⁻⁸ M C5a. The inactive metabolite lyso-PAF had no effect in this system (not shown).

As shown in Fig. 4A, sequential activation with the same agonist did not induce an increase in intracellular calcium, even when the time period between the first and second stimulation was more than 1 h. However, when eosinophils were washed using complete culture medium after the first PAF stimulation, cells responded to second stimulation with the same ligand already after 5 min with a calcium rise (Fig. 4B). In contrast, if cells were washed in medium containing 10⁻⁷ M PAF, no second calcium response was observed (not shown), implying that washing with medium alone

![Table 1. Repetitive PAF-induced ECP release (micrograms per liter) from GM-CSF primed eosinophils.](Image)
might have removed PAF from its receptor (see below). The investigation of shorter time periods between stimulations was technically impossible. In contrast to the PAF experiments, the second C5a response was abrogated within the first 5 min after initial stimulation. However, significant increases in cytosolic free calcium were observed when the second C5a stimulation was performed 15 min after the first stimulation (Fig. 4B). C5a stimulations at later time periods (up to 1 h) did not give higher responses (not shown).

**PAF stimulation does not result in a complete loss of PAF receptors on the surface of eosinophils**

To understand the responsiveness of eosinophils toward the same agonist following a washing step, we performed ligand-binding studies. Using fluorescent PAF and fluorescent lyso-PAF (which also binds to the PAF receptor), we performed flow cytometric and microscopic studies. As shown in Fig. 5A, fluorescent PAF bound to freshly purified eosinophils confirming earlier studies on the presence of PAF surface receptors on these cells (12). The signal was blocked by the specific PAF receptor antagonist WEB 2086, suggesting that the majority of fluorescent PAF binding likely occurred via PAF surface receptors. Moreover, washing the cells resulted in a complete loss of the signal when they were incubated at 37°C but not at 4°C. Reapplication of the fluorescent probe following the washing procedure confirmed the availability of receptors following the washing procedure. The number at the upper right corner of each histogram indicates the ratio calculated as the mean channel fluorescence level following PAF staining divided by the mean channel fluorescence level obtained from unstained eosinophils. In the presence of WEB 2086 (1 mM), fluorescent PAF binding was blocked (ratio: 0.96). Results are representative of three independent experiments. B. Fluorescent microscopy. Fluorescent lyso-PAF bound to eosinophil membrane. Washing removed the fluorescent ring signal. A second incubation with fluorescent lyso-PAF resulted again in a ring-like staining of the eosinophils, suggesting the availability of free PAF receptors. Similar results were obtained using fluorescent PAF, although the fluorescent signal was slightly decreased compared with lyso-PAF. Results are representative of three independent experiments.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Freshly Purified</th>
<th>Medium (4 h)</th>
<th>GM-CSF (4 h)</th>
<th>Medium (8 h)</th>
<th>GM-CSF (8 h)</th>
<th>Medium (20 h)</th>
<th>GM-CSF (20 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1623</td>
<td>1910</td>
<td>1750</td>
<td>1962</td>
<td>1886</td>
<td>1968</td>
<td>1816</td>
</tr>
<tr>
<td>2</td>
<td>2110</td>
<td>2160</td>
<td>2000</td>
<td>2120</td>
<td>2040</td>
<td>2120</td>
<td>1906</td>
</tr>
<tr>
<td>3</td>
<td>2410</td>
<td>2740</td>
<td>2860</td>
<td>2800</td>
<td>2900</td>
<td>2860</td>
<td>2760</td>
</tr>
</tbody>
</table>

*Eosinophils were cultured in the absence or presence of GM-CSF for the indicated times. Total ECP content was determined in Triton X-100-treated eosinophils (10⁶/ml). Three independent experiments are shown.
with labeled PAF at 4°C, indicating that fluorescent PAF was removed by this procedure. In contrast, a small remaining signal was observed when eosinophils were exposed before washing to fluorescent PAF at 37°C, implying at least partial internalization of ligand/receptor complexes (23). When cells were incubated a second time with fluorescent PAF, a strong signal was seen, independent from the temperature of incubation.

Similar results were obtained using fluorescent microscopy. Incubation of eosinophils with fluorescent PAF or fluorescent lysopAF resulted in a ring-like signal, suggesting that these reagents bound to PAF surface receptors (Fig. 5B). Washing of the cells removed the fluorescent ring. A second incubation with the fluorescent agonist generated again a signal mainly focussed on the cellular plasma membrane. Taken together, these data suggest that some PAF/PAF receptor complexes are internalized following PAF stimulation. However, a significant proportion of PAF receptors remains on the surface and is immediately available for second stimulation as long as the agonist from the previous stimulation has been removed.

GM-CSF does not induce ECP gene expression in eosinophils

We next investigated whether the eosinophil priming and survival factor GM-CSF could influence ECP gene expression. As shown in Table II, GM-CSF stimulation of blood eosinophils up to 20 h did not alter the total ECP content compared with freshly purified cells. Moreover, GM-CSF did not appear to increase ECP mRNA expression as assessed by a nonquantitative RT-PCR technique (Fig. 6A). We also investigated the effect of GM-CSF on ECP gene expression in eosinophils after the release of significant amounts of ECP. GM-CSF primed eosinophils were simultaneously stimulated with optimal doses of PAF and C5a up to three times. The resulting cell populations after three stimulations had in average 48.5% of the original ECP content (Fig. 7, mean ± SEM of total cellular ECP levels of five independent experiments: unstimulated cells 2063 ± 136 μg/L, after stimulation 1001 ± 102 μg/L) and were 80–95% viable. GM-CSF did not appear to increase ECP mRNA (Fig. 6B) or protein (Fig. 7) expression in such eosinophils with decreased ECP content.

ECP expression in tissue eosinophils

Because we had no evidence for induction of the ECP gene by the eosinophil survival factor GM-CSF, we investigated whether eosinophils might be depleted of ECP under certain conditions of inflammation. ECP expression was studied in nasal mucosa, gastrointestinal tract mucosa, dermis, bladder cancer, and bone (Fig. 8). In each case, eosinophils strongly expressed ECP. Moreover, extracellular deposition of ECP was frequently observed, suggesting eosinophil activation and ECP release in vivo. In addition,
supernatants from nasal polyp tissues cultured ex vivo contained up to 96 μg/l ECP. Furthermore, indicating eosinophil activation in eosinophilic inflammatory tissues (not shown). The fact that eosinophils always contain detectable amounts of ECP in vivo (and in vitro, even after repetitive stimulation) indicate that eosinophils may always have sufficient amounts of this protein available and therefore may not require additional activation of the ECP gene.

**Discussion**

Eosinophils are inflammatory effector cells associated with various allergic and parasitic diseases as well as malignant disorders (24). The process of eosinophil apoptosis appears to be delayed in eosinophilic tissues and this likely contributes to tissue eosinophilia (9). The purpose of this study was to investigate whether an eosinophil can be repetitively stimulated by the same agonist, a mechanism, which is likely to occur in long-living eosinophils in vivo.

As an in vitro model for eosinophil degranulation, we used peripheral blood eosinophils primed with GM-CSF. It has previously been shown that IL-3, IL-5, and GM-CSF strongly enhance the response of eosinophils to different agonists (3–6, 8). We therefore used peripheral blood eosinophils primed with GM-CSF as a model of eosinophil degranulation. In agreement with previously published work, GM-CSF alone did not stimulate eosinophils for ECP release (25). After optimal stimulation with PAF or C5a, primed eosinophils released large amounts of ECP into the supernatant. In this ECP release assay, GM-CSF changed the eosinophil function in a qualitative manner, because both PAF and C5a alone had no effect.

Whereas previously published work suggested that eotaxin induces ECP release from eosinophils through the activation of extracellular signal-regulated kinase-2 and p38 mitogen-activated protein kinase (26), we did not observe eotaxin-induced ECP release in both untreated and GM-CSF primed eosinophils. It is likely that different experimental conditions are responsible for these apparently controversial results. For instance, Kampen et al. (26) cultured the cells in RPMI 1640 containing 0.1% human serum albumin and stimulated for 4 h. Our experience is that, under such conditions, at least a subpopulation of eosinophils adheres to the plastic surface and might additionally be activated via adhesion molecules (our unpublished observations). We used RPMI 1640 supplemented with 10% FBS and stimulated for 45 min, a condition where adhesion of eosinophils does unlikely occur. Takafulji et al. (27) used cytochalasin B in in vitro eosinophil degranulation experiments. Our system did not require cytochalasin B pretreatment. In fact, we found that cytochalasin B, a drug that destroys assembly of cytoplasmic microfilaments, is toxic and therefore not suitable for eosinophil degranulation assays.

Similar to earlier studies where eosinophils were stimulated by chemokines (28), we found that PAF- or C5a-induced calcium responses were attenuated following previous stimulation with the same agonist in vitro. Several mechanisms may be responsible for abrogation of the second response, including receptor occupancy, down regulation due to internalization, or uncoupling from downstream effector mechanisms. In our experiments, we found that upon washing the cells, the full recovery of the PAF response to repeated application of the ligand takes some 20 min. This probably excludes receptor occupancy as a sole mechanism for the desensitization. Receptor internalization (23) and recycling may take some time, which is in agreement with our findings. In addition, there appears to be a general decline of the released ECP amount after multiple activation events (Table I), also indicating that besides receptor occupancy other desensitization mechanisms may occur. Indeed, we found some degree of internalization at 37°C. A significant proportion of PAF receptors were, however, not internalized. Thus, this mechanism is unlikely to account for the complete loss of the response upon repeated application of the ligand. In our study, we found that receptor occupancy induces long-lasting changes in signal transduction, e.g., the diminished calcium signal. Homologous uncoupling of the receptor from downstream effector mechanisms has best been demonstrated in G-protein-coupled receptors (29, 30). In this case, preferential phosphorylation of occupied receptors leads to their inactivation. It also enhances their internalization and indirectly facilitates their recycling. The involvement of this mechanism in PAF-induced desensitization is largely circumstantial: PAF receptor is indeed a G-protein-coupled receptor (31), whose cytoplasmic tail contains a phosphorylation site (32). The removal of this site prolongs the response to receptor activation, indicating that it may be involved in receptor inactivation or uncoupling. Other mechanisms, which may contribute to receptor uncoupling may involve phosphorylation of phospholipase Cβ, downstream from the receptor activation step (32).

After C5a stimulation of eosinophils, we observed a complete unresponsiveness in a short period of time as well as reduced calcium rises and less released ECP at later time points. This suggests that the proportion of ligand-induced receptor internalization may play a larger role in this compared with the PAF system, or that under our experimental conditions the efficiency of the washing step was lower in the C5a compared with the PAF system. Nevertheless, a significant proportion of C5a receptors is still available for a rapid second stimulation as soon as the previous ligand has been removed.

The possibility to immediately resensitize eosinophils following PAF stimulation enabled us to stimulate them for at least six times within a few hours with the same agonist. Each time, agonist-mediated stimulation resulted in the release of significant amounts of ECP. Following such repetitive stimulation, the ECP content was in average 57% of the level observed in unstimulated cells. Because no desensitization even in the absence of a washing step was observed when eosinophils were first stimulated with PAF and subsequently activated with C5a (or vice versa), we used both reagents for further and more rapid reduction of cellular ECP contents. When eosinophils were simultaneously stimulated with PAF and C5a for three times, they contained in average 48.5% of the original ECP content.

Eosinophils with decreased ECP levels served as a model to investigate the question whether eosinophils are able to increase ECP production. Neither eosinophils with decreased nor normal ECP levels demonstrated evidence for induction of the ECP gene in response to long-term GM-CSF stimulation. Although both PAF and C5a did also not increase the cellular ECP content in this experimental in vitro model (not shown), our data do not exclude the possibility that other cytokines or soluble factors may induce the ECP gene under these or other conditions. For instance, it has been demonstrated that ECP is produced in immature eosinophils in the bone marrow (33). Moreover, it appeared that ECP levels are high in mature eosinophils, suggesting that it may not or only rarely occur that eosinophils do not have sufficient ECP levels even after repetitive stimulation. Our finding that eosinophils always expressed detectable amounts of ECP in eight different eosinophilic inflammatory tissues support this idea.

**References**


