

Mechanisms of human eosinophil activation by complement protein C5a and platelet-activating factor: similar functional responses are accompanied by different morphologic alterations

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The complement system is an important amplification system for the propagation of allergic as well as pseudoallergic inflammatory reactions. In the present study, the effect of the major anaphylatoxin C5a was compared with that of platelet-activating factor (PAF) on highly purified eosinophils ($\geq 95\%$) by functional as well as morphologic criteria. Upon stimulation with C5a, eosinophils maintained their spheric structure, developing short, pseudopodia-like protrusions, whereas PAF induced the generation of a number of digitating protrusions. As shown by functional and ultrastructural assay systems, both stimuli provoked significant extracellular and intracellular H_2O_2 production in eosinophils, which was inhibited by cytochalasin B. With C5a, a pronounced H_2O_2 production was detected within the small cytoplasmic vesicles, whereas PAF-induced H_2O_2 production was observed on the outer surface of the plasma membrane in the contact zones between adjacent cells. Morphologic signs of degranulation induced by C5a and PAF were accompanied by the significantly increased release of eosinophil cationic protein and eosinophil peroxidase in the presence of cytochalasin B. Like PAF, C5a induced a significant production of reactive oxygen species in eosinophils, as measured by lucigenin-dependent chemiluminescence (CL) responses in eosinophils. Maximal responses, comparable with those of interleukin-5 (100 U/ml), were observed at concentrations of 10^{-5} – 10^{-6} and 10^{-7} – 10^{-8} M for PAF and C5a, respectively. Separation of eosinophils by discontinuous density gradients revealed the existence of two hypodense eosinophil populations, one of them showing significantly reduced CL responses upon stimulation with C5a and PAF. In addition, CL responses upon stimulation with C5a and PAF were abrogated by cytochalasin B, staurosporine, and wortmannin, and were almost completely blocked by pertussis toxin. In conclusion, these data indicate that C5a induces events in human eosinophils comparable to those induced by PAF in the assay systems tested. Thus, C5a, generated after activation of the complement system, may be of major importance for the eosinophil activation observed in eosinophil-related disease.

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Eosinophils have been implicated in the pathogenesis of various allergic and nonallergic diseases such as bronchial asthma, atopic dermatitis, parasitosis, and certain interstitial pulmonary diseases (52). In these disorders, eosinophils are considered to be im-

portant effector cells which can cause local damage to infiltrated tissue via the release of reactive oxygen species (ROS) and a number of cytotoxic basic proteins stored in their cytoplasmic granules (18). Evidence of the detrimental effect of eosinophils in dis-

ease derives from the association of tissue destruction with an accumulation of eosinophils, deposits of eosinophil-derived products, or both *in vivo* (4, 15). In addition, several *in vitro* studies have shown that activated intact eosinophils (60) and both eosinophil-derived basic proteins and oxygen radicals kill helminthic parasites and destroy tumor cells and other mammalian cells, including guinea pig epithelium (52).

Eosinophils found in active disease, such as asthma (6, 7) and atopic dermatitis (5, 23), show characteristic ultrastructural changes that include formation of cytoplasmic vacuoles, alterations in cell size and shape, increase in granule size, solubilization and loss of granular core and matrix proteins, and increase in vesiculotubular structures (11, 34, 43), all of which can also be induced by *in vitro* cell activation (11, 27, 34, 59). Hence, these ultrastructural alterations observed in eosinophils are probably caused by mediators produced by other participating cells or by complement proteins released during inflammatory processes.

Although several mediators with potential eosinophil-stimulating properties have been identified, their individual contribution to eosinophil activation both *in vivo* and *in vitro* has yet to be delineated. Among them, lipid mediators such as leukotriene (LT) B_4 or platelet-activating factor (PAF), cytokines, immunoglobulins, and tachykinins (2) have been shown to stimulate various eosinophil functions. In addition, complement proteins, such as C5a and C3b (assessed with opsonized parasites, zymosan, or sepharose) have been implicated as eosinophil activators that facilitate chemotaxis (i.e., C5a) and binding of eosinophils to large surfaces, thereby inducing secretion of granular contents (14, 21, 30, 41, 48, 52, 58). A number of receptors for the complement factors C1q, C3b/C4b (CRI), iC3b (CRIII), and C3d have been identified on human eosinophils (1). Recently, researchers identified a receptor for C5a on eosinophils which differs from the C5a receptor on neutrophils in both structure and affinity (16). However, while the effect of PAF has been extensively studied (3), little information is available on eosinophil responses after exposure to C5a and, particularly, on the significance and relative potency of C5a with respect to other eosinophil-activating factors. Likewise, to our knowledge, the signaling pathway of C5a has so far not been investigated in detail.

Studies published to date have mostly focused on functional responses of the respective activators, while data on the accompanying morphologic changes are rare despite the possibility that parallel evaluation of both functional and morphologic responses may provide additional insight into the mechanisms of eosinophil activation and disclose potential discrepancies in cellular responses to dif-

ferent stimuli. Hence, the purpose of the present study was, firstly, to examine the mechanism of eosinophil response to the complement protein C5a on an ultrastructural level by both transmission and scanning electron microscopy (TEM, SEM), and, secondly, to relate those findings to the functional performance of eosinophils as measured by the production of ROS and the release of toxic granule proteins. Furthermore, for assessment of the relative efficiency of C5a, its effects were compared with those obtained by stimulation with PAF.

Material and methods

Stimuli and cytokines

Escherichia coli-derived recombinant human C5a and β -acetyl- γ -*o*-hexadecyl L- α -phosphatidylcholine (PAF) was obtained from Sigma, Munich, Germany. A stock solution of 10^{-2} M PAF was prepared in ethanol/DMSO 50:50 (v/v) and stored at -70°C . Dilutions were made in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA).

E. coli-derived recombinant human tumor necrosis factor- α (TNF- α) with a specific activity of 8.5×10^6 U/mg was provided by BASF, Ludwigshafen, Germany. Recombinant human interleukin (IL)-8/NAP-1 was a kind gift from the Sandoz Forschungsinstitut, Vienna, Austria. Recombinant human granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-2, IL-3, IL-5, and interferon- γ (IFN- γ) were purchased from Genzyme, Boston, MA, USA. Before testing, all cytokines were diluted in PBS containing 50 $\mu\text{g}/\text{ml}$ BSA.

Reagents and antibodies

Ficoll-Paque solution was obtained from Pharmacia (Freiburg, Germany); purified BSA, lucigenin, luminol, phorbol-myristate-acetate (PMA), horseradish peroxidase type I, catalase, and cytochalasin B were obtained from Sigma (Munich, Germany). Staurosporine and pertussis toxin were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). Culture media and antibiotics were purchased from Biochrom-Seromed (Berlin, Germany). CeCl_3 was obtained from Fluka (Buchs, Switzerland). Reagents and media for electron microscopy were obtained from Roth (Karlsruhe, Germany). A monoclonal mouse IgG2a against CD16 (BW 209/2) was a gift from Dr R. Kurrle, Behring Werke (Marburg, Germany). The PAF-antagonist (Ro 24-0238) was donated by Dr F. Schneider, Hoffmann-LaRoche (Basel, Switzerland). Wortmannin was kindly supplied by Dr D. Römer, Sandoz (Basel, Switzerland). An inhibitory monoclonal mouse- α C5a IgG1 (C17/

5), cross-reacting with a neoepitope of the C5a molecule, was graciously provided by Prof. O. Götze, Department of Immunology, University of Göttingen, Germany.

Isolation of human granulocytes

Human granulocytes were isolated from heparin-anticoagulated venous blood of normal nonatopic blood donors, as described previously (25). For further purification of eosinophils, granulocytes were resuspended at a concentration of 10^7 /ml in HEPES-buffered Hanks' balanced salt solution, pH 7.4, containing 1 mg/ml BSA (HBSS/BSA).

Purification of human eosinophilic granulocytes

Eosinophils were purified as described previously (28). For this purpose, immunomagnetic beads (Dynabeads M-450, Dynal, Norway) were coated with monoclonal α CD16. Briefly, 2 ml beads (4×10^8 beads/ml) was mixed with 50 μ l α CD16 (1 mg/ml) and incubated for 24 h at 4°C in Minisorp tubes (Nunc, Roskilde, Denmark) on a rotary mixer. Coated beads were washed three times in HBSS/BSA, and the beads were retrieved with a Dynal magnetic particle concentrator (MPC-6). α CD16-coated beads were stored at a concentration of 2×10^8 beads/ml HBSS/BSA under sterile conditions for a maximum of 1 week at 4°C. A volume of 1 ml granulocytes was centrifuged in Minisorp tubes for 7 min/820 g at 4°C, the supernatant was aspirated, and later 500 μ l of the α CD16-coated beads was added to the pellet. The mixture was incubated for 1 h at 4°C on a rotary mixer. Thereafter, HBSS/BSA was added and polymorphonuclear neutrophilic granulocytes (PMN), which were coupled to the beads, were removed magnetically with the MPC-6 device. Supernatant was aspirated and residual beads were removed by a second magnetic separation step. The resulting supernatant was washed as described above, and the resulting eosinophils were resuspended in HBSS/BSA. When necessary, the magnetic purification procedure was repeated once more. Eosinophils were quantitated with Kimura stain (32) in a Neubauer counting chamber. Cytospin preparations of the cells had a purity of over 95%, as shown by Pappenheim stain.

Separation of eosinophils on discontinuous Percoll gradients

Highly purified eosinophils were further separated by centrifugation on Percoll (Kabi Pharmacia, Freiburg, Germany) density gradients (43). For this purpose, five-step discontinuous density gradients

(750 μ l of each interface per gradient) were formed with a peristaltic pump (Vario-perpex II pump, Kabi-Pharmacia, Freiburg, Germany) in 5-ml, round-bottom polystyrene tubes. In brief, gradients consisted of 1.08, 1.085, 1.09, and 1.1 g/ml isotonic buffered Percoll solution (43). Purified as described above, 5×10^6 eosinophils were washed twice and resuspended in 750 μ l Percoll solution (density of 1.07 g/ml). Percoll gradients were overlaid with these cells and centrifuged for 20 min/1600 g at 10°C. Thereafter, the cells in the interphases were collected, immediately washed twice with HBSS/BSA, and resuspended as described below. Eosinophils with a density of less than 1.082 g/ml were defined as hypodense (59). Cells were stained and counted as described above. There were no differences in viability of the cells (92–95%) between the fractions as judged by trypan blue exclusion.

Lucigenin-dependent chemiluminescence (CL)

Lucigenin-dependent CL (26, 27), in contrast to the luminol-enhanced reaction, is independent of the release of peroxidase (13) and is an extremely sensitive measure of the release of ROS from granulocytes, particularly the release of the superoxide anion, because $\geq 70\%$ of the signal could be blocked by superoxide dismutase (26). For simultaneous measurement of large samples (dose-response relations), a single-photon imaging system with a two-dimensional photon-counting system (MTP reader, Hamamatsu Photonics, Herrsching, Germany) was used, as described elsewhere (37, 38). In brief, eosinophils were suspended to a density of 5×10^4 cells/ml in HEPES-buffered Hanks' balanced salt solution (HBSS), pH 7.4, containing 200 μ M lucigenin and 1 mg/ml BSA. Aliquots (100 μ l) containing 5×10^3 cells each were placed in flat-bottom, white microtiter plates (Microfluor, Dynatech Deutschland, Denkendorf, Germany). Measurements were done in triplicate at 37°C. Integral counts from a 0–60-min incubation interval after addition of stimuli to the eosinophils were measured and are indicated as intensity integral counts $\times 10^{-3}$ in figures and tables.

For CL time kinetic studies, eosinophils were suspended to a density of 2.5×10^5 cells/ml in HEPES-buffered Hanks' balanced salt solution (HBSS), pH 7.4, containing 200 μ M lucigenin and 1 mg/ml BSA. Aliquots (200 μ l) containing 5×10^4 cells each were placed in unsealed polystyrene luminescence tubes (Berthold, Wildbad, Germany) and stored at 4°C for a maximum of 2 h before use. Measurements were done at 37°C in two six-channel Biolumat LB 9505C (Berthold, Wildbad, Germany). Integral counts from a 0–60-min incubation interval after addition of stimuli to the granulocytes were

measured and are indicated as counts $\times 10^{-6}$ in figures and tables.

Extracellular and intracellular production of H_2O_2

Extracellular and intracellular production of H_2O_2 was determined with a modification of a sensitive CL method described previously (35, 36). In brief, 50 μ l eosinophils at a concentration of 2×10^5 /ml HBSS/BSA was distributed into flat-bottom, white microtiter plates, and 50 μ l of the reaction mixtures was added and incubated with cells for 20–30 min at 37°C. Subsequently, stimuli or medium as a control was added. Measurements were done in triplicate at 37°C. Integral counts from a 0–60-min incubation interval after addition of stimuli to the eosinophils were measured and are indicated as intensity integral counts $\times 10^{-3}$ in figures and tables. The reaction mixtures for measurement of extracellular H_2O_2 production consisted of 2 mM NaN_3 , 9 U/ml HRPO type I, and 10^{-6} M luminol with or without addition of 10 μ g/ml cytochalasin B, whereas the cocktail used for the detection of intracellular H_2O_2 production contained 4×10^3 U/ml catalase and 10^{-6} M luminol with or without addition of 10 μ g/ml cytochalasin B.

Release of eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP)

For measurement of EPO and ECP release, eosinophils were seeded at a concentration of 5×10^5 /ml HBSS/BSA in 96-well, V-bottom tissue-culture plates with 5×10^4 cells/well. After preincubation of the eosinophils for 5 min at 37°C in the presence or absence of 5 μ g/ml cytochalasin B, stimuli or medium was added. Thereafter, eosinophils were incubated for 30 or 120 min at 37°C. Then incubation plates were centrifuged for 5 min/600 g at 4°C, and supernatants were removed carefully. Subsequently, 100 μ l 0.3% CETAB was added to each 100 μ l supernatant and the mixtures were immediately frozen at -70°C until testing. For EPO measurement, a highly sensitive, enhanced luminescence assay was used. In brief, 100 μ l ECL[®] immunoassay signal reagent free of NaN_3 (Amersham, Braunschweig, Germany) was added to 10 μ l supernatant in flat-bottom, white microtiter plates. Measurements were done in triplicate for 30 min at 37°C. EPO activity contained in the supernatants is expressed as intensity integral counts $\times 10^{-3}$ in figures and tables. Interassay reproducibility was tested by standard curves with serial dilutions of eosinophil lysates (50%–0.5% of total content). Eosinophil lysates were generated as follows: 200 μ l 0.6% CETAB was added to 100 μ l eosinophils at a concentration of 5×10^5 /ml, the mixture was incubated for 60 min at

room temperature on a shaker and centrifuged for 5 min/600 g at 4°C, and the supernatant was immediately frozen at -70°C .

ECP concentration in the supernatants was determined by a commercially available double-antibody radioimmunoassay (ECP-RIA, Kabi-Pharmacia, Uppsala, Sweden) (24).

Ultrastructural detection of eosinophil activation

For assessment of the effect of stimuli on eosinophils by ultrastructural criteria (29, 61), eosinophils were incubated at a concentration of 1×10^6 /ml in microtubes for TEM and SEM, respectively. Thereafter, stimuli or medium was added to the eosinophils. Immediately after addition of the stimuli, 30- μ l aliquots of the mixture were transferred to Bio-Rad adhesion slides (Bio-Rad, Munich, Germany) and incubated for 15 or 30 min at 37°C. Thereafter, eosinophils were fixed for TEM or SEM as described below (29, 61). In brief, eosinophils were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 60 min at 4°C.

For detection of hydrogen peroxide production, eosinophils were incubated at a density of 1×10^6 /ml with stimuli or medium in microtubes for 30 min at 37°C, as previously described (27). Briefly, after centrifugation, unfixed cells were preincubated for 10 min at 37°C in 0.1 M Tris-maleate with 7% sucrose, pH 7.5. The final incubation medium consisted of 0.1 M Tris-maleate with 7% sucrose, pH 7.5, and 1 mM $CeCl_3$. Eosinophils were incubated for 30 min at 37°C in this medium and later washed in sodium cacodylate buffer, pH 6.0, with 5% sucrose, for 60 min at 4°C.

Generally, eosinophils were washed overnight in 0.1 M sodium cacodylate buffer, pH 7.3, with 5% sucrose, postfixed in 1% osmium tetroxide in the same buffer for 60 min, dehydrated in graded ethanols, and embedded in Araldit. Thin sections were routinely stained with 4% aqueous uranyl acetate and lead citrate and examined in a Zeiss EM109.

For SEM, eosinophils were fixed in glutaraldehyde and postfixed in osmium tetroxide, as described above. Samples were dehydrated in a graded series of ethanol (30–100%), critical-point dried, gold-coated, and examined in a Jeol JSM-35CF scanning microscope.

Statistical analysis

Unless otherwise stated, the data in the text and figures are expressed as mean $\pm$ standard error of the mean (SEM). All data were analyzed nonparametrically with the Mann-Whitney U-test. Significance was accepted for $P \leq 0.05$.

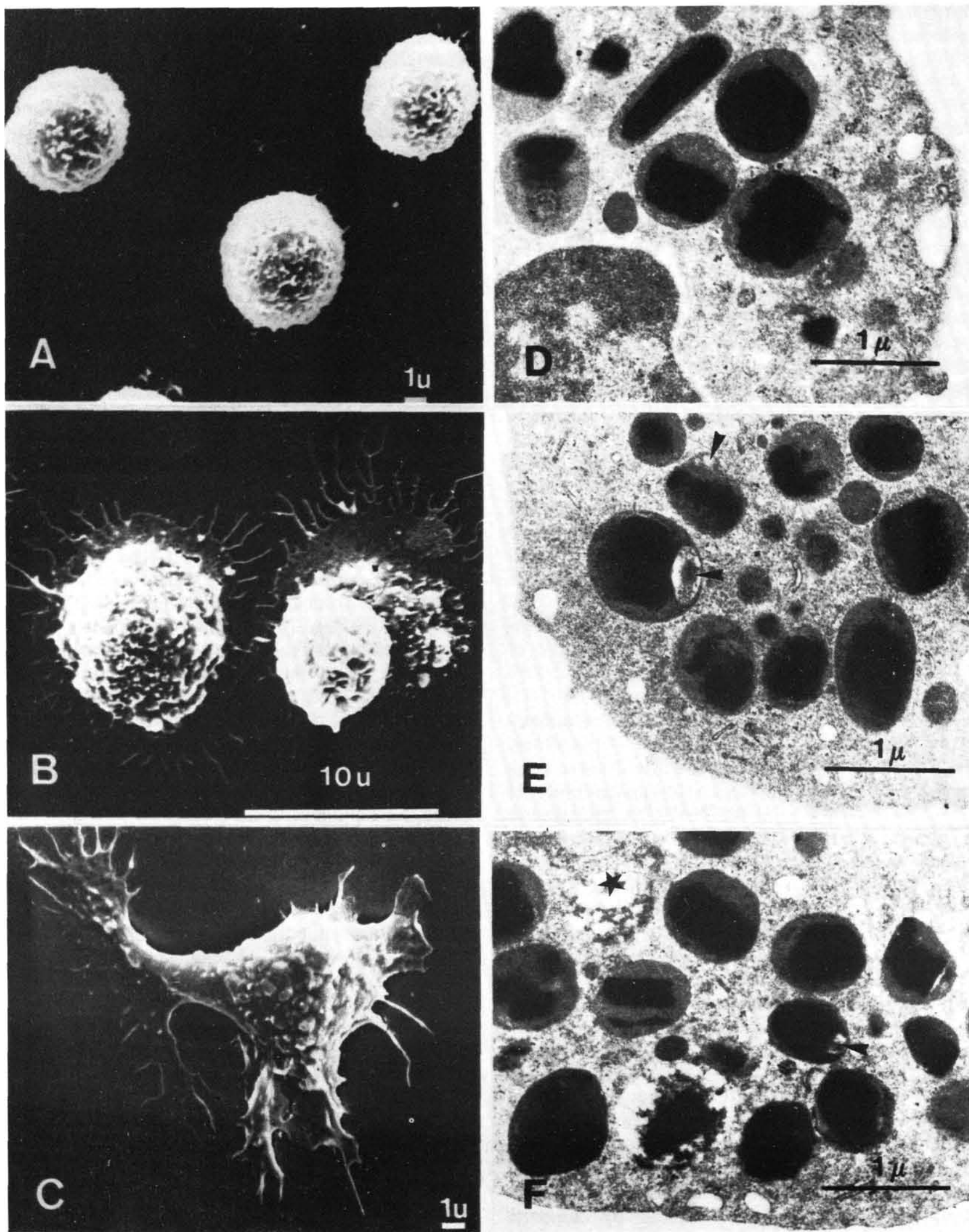


Fig. 1. Effect of C5a and PAF on structure of human eosinophils. Ultrastructural detection of eosinophil activation induced by recombinant human C5a and PAF. A) Unstimulated eosinophils: medium was added instead of stimuli, 15 min at 37°C, SEM; B) eosinophils stimulated with C5a (10^{-7} M), 15 min at 37°C, SEM; C) eosinophils stimulated with PAF (10^{-6} M), 15 min at 37°C, SEM; D) unstimulated eosinophils: medium was added instead of stimuli, 30 min at 37°C, TEM; E) eosinophils stimulated with C5a (10^{-7} M), 30 min at 37°C, TEM; arrow: some secondary granules had lost portions of normally dense matrix, vesiculotubular system

Results

Effect on the structure of human eosinophils (Table 1)

As estimated by SEM, unstimulated eosinophils were found to be rounded, with a ruffled surface membrane (Fig. 1A). Upon stimulation with C5a, eosinophils maintained their spheric structure, surrounded with very small surface microridges and tiny pseudopodia-like protrusions (Fig. 1B). In contrast, PAF-stimulated cells appeared to be flattened; characteristically, a number of digitating, pseudopodia-like protrusions deriving from the main cell body were seen (Fig. 1C).

As estimated by TEM, unstimulated eosinophils revealed dense, specific granules displaying centrally located crystalloid cores and an outer matrix, all bounded by a typical granule membrane (Fig. 1D). Only a few, small, membrane-bound, dense granules and some vesiculotubular structures were seen. Golgi vesicles, mitochondria, and other organelles appeared to be normal. After addition of C5a, eosinophils showed characteristic signs of activation. Some secondary granules of the eosinophils had lost portions of the normally dense matrix. In addition, C5a induced the appearance of vesicular structures in the matrix of the specific granules (Fig. 1E). Adjacent granule matrices interconnected, and two crystalloid cores were detected within combined structures. Moreover, increased numbers of small, electron-dense, cytoplasmic granules were seen. Many electron-light vesicles were found to be derived from the strongly activated Golgi apparatus. The vesiculotubular system had a strongly pronounced appearance, particularly in C5a-stimulated eosinophils.

In contrast, PAF induced characteristic changes of secondary granules. Some secondary granules had lost portions or all of the normally dense matrix. In a number of cells, unchanged dense cores remained in an otherwise empty membrane-bound granule. Furthermore, large empty vesicles could be detected (note * in Fig. 1F). Upon stimulation with PAF, more small, dense granules were seen; these were significantly more numerous than in eosinophils stimulated with C5a. The vesiculotubular system was markedly expressed in these cells, and microgranules were observed.

More cytoplasmic lipid bodies were observed in both C5a- and PAF-stimulated eosinophils than in unstimulated cells.

Table 1. Ultrastructural features of C5a- and PAF-stimulated eosinophils

Ultrastructural features	C5a (10^{-7} M)	PAF (10^{-6} M)	Medium alone
Shape change (SEM)	Rounded cells, pseudopodia-like protrusions	Flattened cells, digitating, pseudopodia-like protrusions	Spheric cells
Loss of matrix in specific granules	2 ^{a)}	3	0
Small dense granules	2	3	0–1
Electron-light vesicles	4	3	1
Lipid bodies	2	2	1
Detection of H ₂ O ₂	Intracellular	Intra- and extracellular	Not observed

^{a)} Semiquantitative grading (0–4) of morphologic changes and frequency of their occurrence.

Effect on intracellular and extracellular production of H₂O₂

A significant extracellular H₂O₂ production was detected by functional assay systems upon stimulation with C5a < PAF < IL-5 (Fig. 2A). In parallel, a significant intracellular production of H₂O₂ could be observed (Fig. 2B). Both intracellular and extracellular production of H₂O₂ induced by PAF and IL-5 were completely blocked in the presence of cytochalasin B (Fig. 2). In the case of C5a, the extracellular response was completely abrogated in the presence of cytochalasin B, whereas a small production of hydrogen peroxide could still be observed intracellularly (Fig. 2B).

Identical results were obtained when hydrogen peroxide production was visualized by an ultrastructural method. Upon stimulation with C5a, the reaction product was mainly observed on the outer surface of the plasma membrane, particularly in the contact zones between adjacent cells, and in the small cytoplasmic vesicles (Fig. 3A). Hydrogen peroxide production appeared to be “focused” on distinct parts of the plasma membrane. However, upon stimulation with PAF, significant amounts of the reaction product were observed at the outer surface of the plasma membrane (Fig. 3B). Extracellular as well as intracellular production of ROS was completely inhibited by cytochalasin B (data not shown).

Effect of cytochalasin B on morphologic changes

Stimulation of eosinophils in the presence of cytochalasin B, which interferes with the contractile microfilament functions (9), abrogated most of the char-

had strongly pronounced appearance; F) eosinophils stimulated with PAF (10^{-6} M), 30 min at 37°C, TEM; arrow: some secondary granules had lost portions or all of normally dense matrix, * empty vesicles could be detected. Eosinophils used in experiments had purity of 98–99%. Experiment is representative of six essentially identical experiments on eosinophils of healthy, nonatopic blood donors.

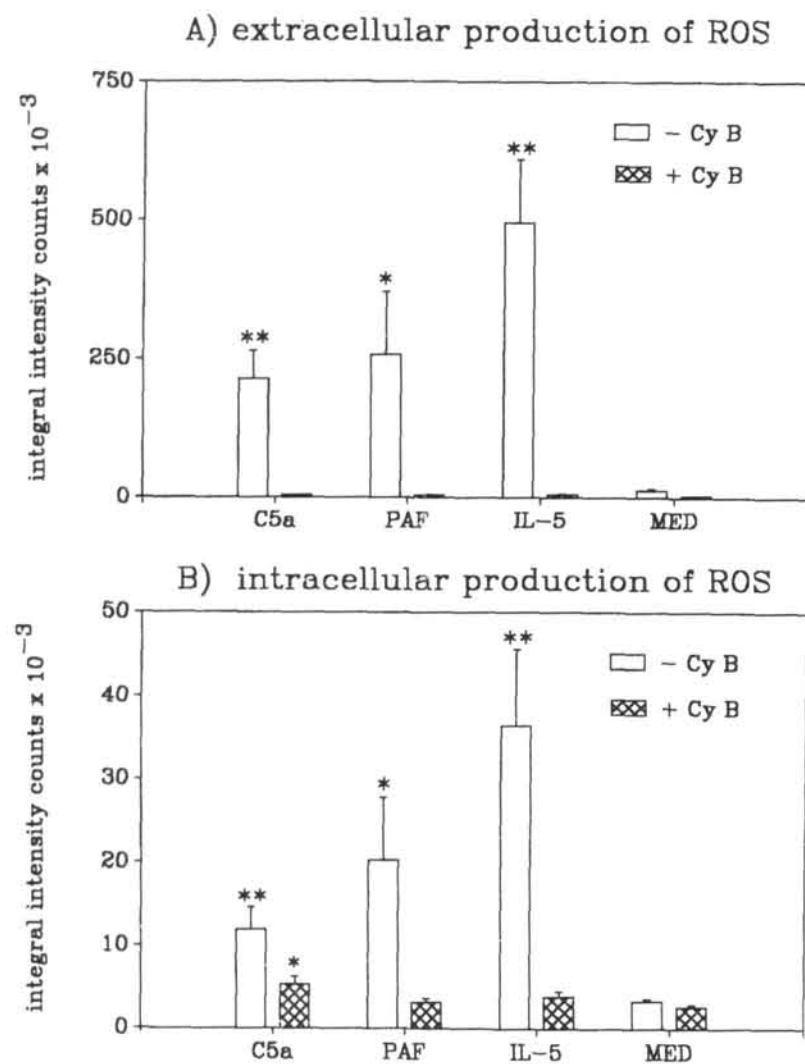


Fig. 2. Detection of extracellular and intracellular production of H_2O_2 by human eosinophils. Effect of cytochalasin B (5 μ g/ml). Recombinant human C5a (10^{-7} M), PAF (10^{-6} M), or IL-5 (10^2 U/ml) were used as stimuli. MED: response induced by addition of medium alone. Values represent mean $\pm$ SEM of six experiments on six subjects.

acteristic morphologic changes induced by C5a or PAF; in particular, no development of dendritic protrusions was seen (Fig. 4A and B). Interestingly, large channels were seen in C5a-stimulated cells treated with cytochalasin B, whereas in PAF-stimulated eosinophils, microridges were found to be preserved on the spheric cell body (Fig. 4B). As estimated by TEM, more electron-light vesicles associated with large channels were seen after stimulation with either C5a or PAF, in cytochalasin B-treated eosinophils (Fig. 4C and D).

Effect on the release of ECP and EPO

C5a significantly stimulated the release of EPO (Fig. 5A) and ECP (Fig. 5B) in the supernatant in the presence of cytochalasin B. In contrast, no relevant effects could be observed in the absence of cytochalasin B. PAF was significantly less active than C5a (Fig. 5). Increase of the incubation time from 60 to 120 min did not cause any significant change in the results (Fig. 5).

Effects of C5a and PAF on the CL response of human eosinophils

Like PAF, C5a induced a significant production of ROS in eosinophils as measured by lucigenin-dependent CL responses in eosinophils. Maximal responses, comparable with those of IL-5 (100 U/ml), were observed at concentrations of 10^{-5} – 10^{-6} M and 10^{-7} – 10^{-8} M for PAF and C5a, respectively (Fig. 6). Higher concentrations of PAF were inhibitory (data not shown).

C5a and PAF induced a typical time course of the CL response, starting immediately after addition of the stimulus, with a peak at about 3–7 min, followed by an increase of base-line activation of the eosinophils for about 1 h (Fig. 7). In contrast, the CL response upon stimulation with IL-5 started after a lag phase of 3–5 min with a peak in the range of 10–15 min, which was similarly followed by a long-lasting increase of base-line activity.

In addition, the effect of PAF and C5a on PMN was investigated in comparison with eosinophils. Upon stimulation with PAF and C5a, eosinophils produced significantly more ROS, as measured by CL, than PMN from identical donors (data not shown). C5a-mediated production of ROS was blocked by a C5a-specific monoclonal antibody (data not shown). Similarly, the presence of the PAF-receptor antagonist Ro 24-0238 specifically inhibited the PAF-induced CL response, whereas no significant effect on C5a-induced CL was observed (data not shown).

Effect on the CL response of hypodense and normodense eosinophils

Separation of eosinophils on discontinuous Percoll gradients revealed at least two hypodense cell populations which were collected at the interphase between 1.07/1.08 g/ml and 1.08/1.085 g/ml (Fig. 8A). Of the cells, $17.4 \pm 1.9\%$ belonged to the population with the lowest density which showed a significantly reduced response to stimulation with C5a, PAF, or IL-5 in comparison with the normodense cell populations (Fig. 8B). In contrast, there were no significant differences in the CL response of the second hypodense cell population ($21.3 \pm 1.8\%$ of the eosinophils) from the normodense eosinophils (Fig. 8B). The viability of the two hypodense cell populations was not different from that of normodense cells, as assessed by trypan blue exclusion.

Effect of inhibitors on CL response of human eosinophils

Pretreatment of eosinophils with pertussis toxin for 120 min at 37°C (31) dose-dependently blocked the

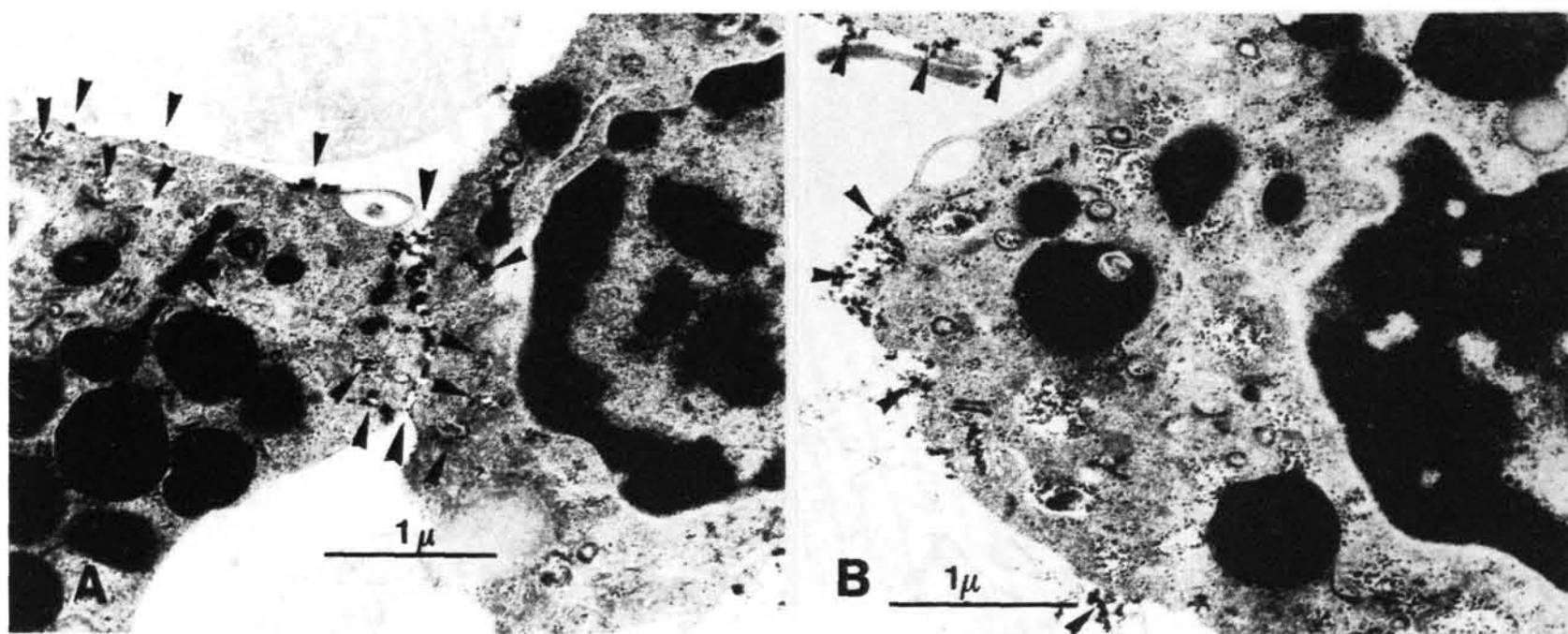


Fig. 3. Ultrastructural detection of H_2O_2 production by human eosinophils upon stimulation with recombinant human C5a and PAF. A) eosinophils stimulated with C5a (10^{-7} M), 30 min at 37°C ; B) eosinophils stimulated with PAF (10^{-6} M), 30 min at 37°C . Arrowheads: production of hydrogen peroxide visualized by detection of electron-dense precipitate of $\text{Ce}(\text{OH})_3\text{OOH}$ (see Material and methods). Experiment is representative of five essentially identical experiments on healthy, nonatopic blood donors.

CL responses induced by C5a or PAF (Fig. 9A), whereas production of ROS induced by IL-5 was strongly suppressed only at higher concentrations of pertussis toxin (Fig. 9A). Pretreatment of the eosinophils with staurosporine, which inhibits PMA-dependent protein phosphorylation and the respiratory burst in human PMN (10), inhibited CL responses upon stimulation with C5a, PAF, or IL-5 dose-dependently. A complete inhibition was observed at a concentration of 100 nM (Fig. 9B).

Pretreatment of the eosinophils with $1\ \mu\text{M}$ wortmannin, which inhibits a step proximal to phospholipase D (1, 44), totally abrogated C5a-induced CL responses (data not shown).

Modulation of C5a- and PAF-induced CL responses by preincubation with cytokines

For assessment of the modulating effect of cytokines on the CL response, eosinophils were preincubated with IL-2, IL-3, IL-5, IL-8, IFN- γ , GM-CSF, and TNF- α for 60 min at 37°C and subsequently stimulated with C5a (10^{-7} M) or PAF (10^{-6} M) (Table 2). However, no significant differences between eosinophils preincubated with cytokines and eosinophils preincubated with medium alone could be detected.

Discussion

The complement cascade consists of a number of proteins which may play a pathogenic role in various disorders, including anaphylactic and pseudoallergic reactions, as well as bronchial asthma, idiopathic pulmonary fibrosis, or adult respiratory distress syn-

drome (ARDS) (8, 39, 46, 49). Components of the alternative pathway of complement activation are present in respiratory secretions (47), as are complement deposits in the bronchial mucosa of asthmatic subjects (8, 39) and complement proteins in the bronchoalveolar fluid in idiopathic pulmonary fibrosis or ARDS (46, 49). Thus, because eosinophils have been implicated in the pathogenesis of these conditions (18–20), production of C5a may be particularly significant. Previous studies have shown that the complement-cascade-derived protein C5a is a potent eosinophil chemoattractant both *in vitro* (30, 40, 45, 48) and *in vivo* (14). The data presented herein confirm these observations and extend the spectrum of stimulatory activities of C5a on human eosinophils to include the intracellular and extracellular production of ROS and cellular protein release, as measured for EPO and ECP. Qualitatively, the functional responses of C5a resembled those of PAF. However, in production of oxygen radicals and release of toxic granule proteins, C5a seemed to be the more potent stimulus. Moreover, although the stimulatory response to the agonists was qualitatively similar and equally sensitive to inhibition by pertussis toxin, staurosporine, and wortmannin, on an ultrastructural level C5a and PAF induced different morphologic alterations in eosinophils. This was shown with highly purified eosinophils of healthy nonatopic blood donors to avoid disease-specific preactivation of the cells (27).

Upon stimulation with C5a, eosinophils maintained their spheric structure. Nevertheless, cells adhered to the glass surface, as shown by the detection of small, pseudopodia-like protrusions in the circumference. In contrast to those stimulated by C5a,

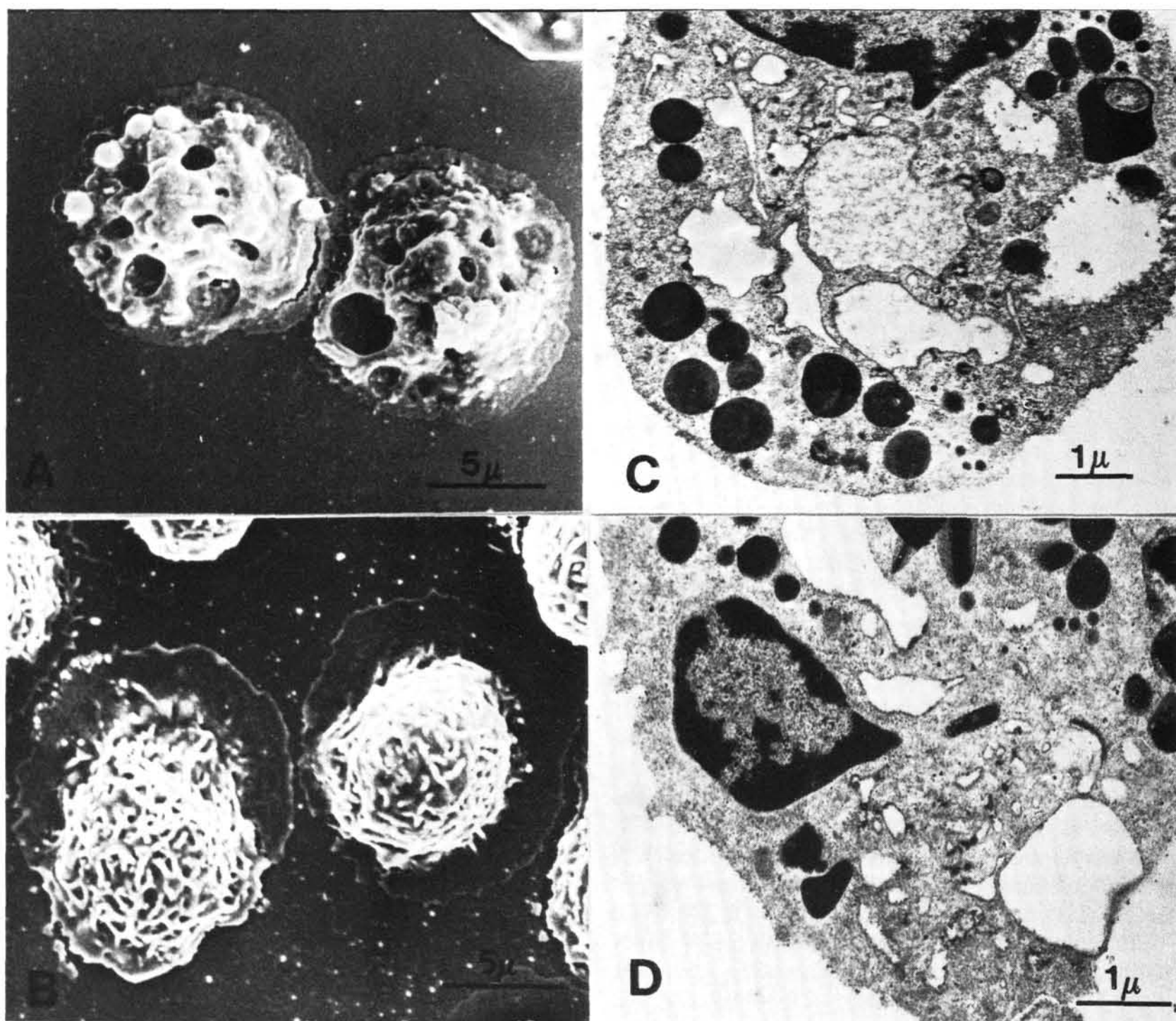


Fig. 4. Effect of cytochalasin B on C5a- and PAF-induced morphologic changes. Eosinophils were pretreated with cytochalasin B (5 μ g/ml) for 5 min at 37°C and later stimulated with A) C5a (10^{-7} M), 15 min at 37°C, SEM; B) PAF (10^{-6} M), 15 min at 37°C, SEM; C) C5a (10^{-7} M), 15 min at 37°C, TEM; and D) PAF (10^{-6} M), 15 min at 37°C, TEM. Experiment is representative of three essentially identical experiments on healthy, nonatopic blood donors.

PAF-stimulated cells developed long pseudopodia, mainly seen at one side of the cell. This typical polarization is characteristic of inflammatory cells responding to chemotactic factors (51). However, there was obviously no remarkable difference in the chemotactic potency between C5a and PAF (45), suggesting that morphologic signs of polarization are not unequivocally associated with the chemotactic activity. All morphologic changes could be blocked by cytochalasin B, indicating the involvement of the actin cytoskeleton in both C5a- and PAF-induced eosinophil activation (9). As estimated by histochemical and functional findings, a localized production of hydrogen peroxide was detected with both C5a and PAF. Whereas C5a-induced production of hydrogen peroxide was focused on distinct parts of the

plasma membrane, that is, exclusively at the contact zones between adjacent cells, the reaction product upon stimulation with PAF was detected at the outer surface of the plasma membrane, particularly at the end of the pseudopodia. However, significant intracellular production of hydrogen peroxide was observed only upon stimulation with C5a and could be localized at the luminal part of the intracytoplasmic vacuoles, representing a characteristic ultrastructural finding of C5a-stimulated eosinophils. Extracellular as well as intracellular production of hydrogen peroxide upon stimulation with C5a or PAF was abrogated in the presence of cytochalasin B. These findings strongly support the view that C5a-mediated functional activation of eosinophils is associated with the morphologic transformation of the cells.

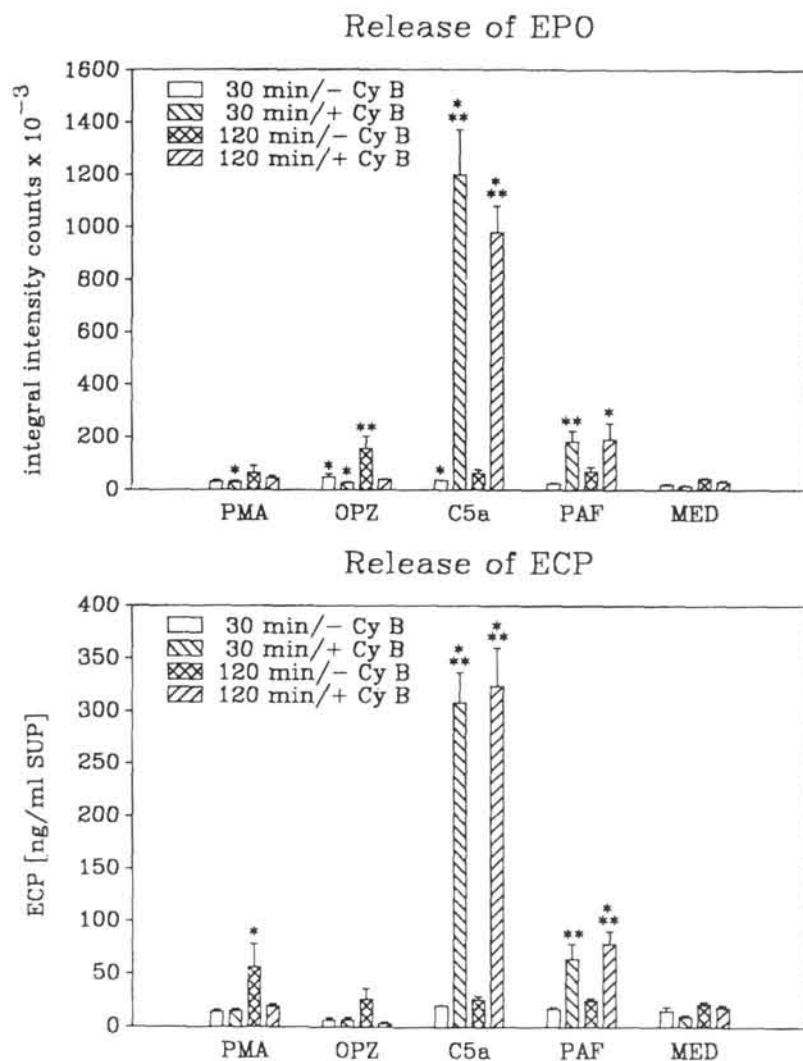


Fig. 5. Release of EPO and ECP by eosinophils of healthy, non-atopic blood donors upon stimulation with C5a and PAF. Eosinophils were stimulated with recombinant human C5a (10^{-7} M), PAF (10^{-6} M), PMA (10 ng/ml), and opsonized zymosan (1 mg/ml) in presence or absence of cytochalasin B (5 μ g/ml) for 60 or 120 min/ 37° C. EPO activity was measured in supernatants by highly sensitive enhanced luminescence assay. ECP concentration was determined by double-antibody radioimmunoassay. MED = spontaneous release after addition of medium alone. Values represent mean $\pm$ SEM of triplicate experiments on five or six healthy, nonatopic blood donors. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, as compared with MED.

C5a and PAF caused distinct morphologic changes, quite like those of activated tissue eosinophils *in vivo*. These included fewer specific granules, more small, dense granules (12), and more large, membrane-bound, lipid bodies (56, 57). In addition, characteristic ultrastructural alterations of eosinophil secondary granules could also be detected for both stimuli. Whereas the fusion of two crystalloid cores was mainly observed in C5a-stimulated cells, the dissolution of the core of secondary granules (the so-called empty membranes) was a characteristic finding in eosinophils after stimulation by PAF (34). Both stimuli consistently induced the formation of tubulovesicular structures in the matrix of specific granules. These structures are thought to be an integral component of the specific granule matrix; they are masked by the packed enzymic contents and disclosed only during granule lysis (42). Moreover, markedly increased numbers of so-called microgran-

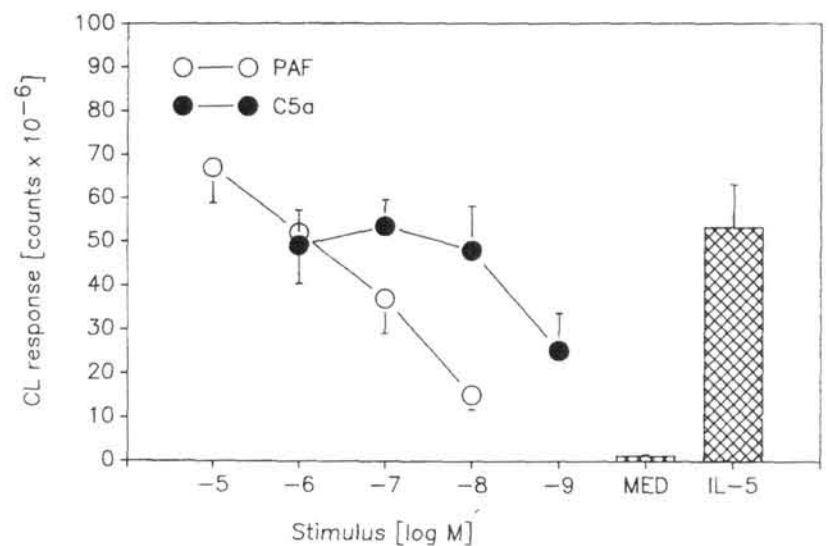


Fig. 6. Effects of C5a and PAF on oxidative metabolism of human eosinophils. CL response of purified eosinophils from normal, nonatopic blood donors: effect of PAF or recombinant human C5a. Values represent mean $\pm$ SEM of experiments on seven healthy, nonatopic controls. Effect of recombinant human IL-5 (100 U/ml) as positive control is shown in parallel. MED: background, induced by addition of medium alone. Integral counts from 0–30-min incubation interval after addition of stimuli were measured (Biolumat LB9505C).

ules, as part of the intracytoplasmic tubulovesicular structure, representing a unique finding for eosinophils (50), were regularly found upon stimulation with C5a. Increased vesiculation upon stimulation with C5a may be an important step in the formation of intracellular vacuoles and channels into which granule components are fully solubilized before extracellular release (21). Particularly in C5a-stimulated cells, pores were noted in the surface membrane, suggesting communication with vacuoles and channels. These structures were also seen in PAF-stimulated eosinophils and may generally aid the release of granule contents into the extracellular space (22).

Microridges were greatly reduced at this stage of secretion. However, the formation of these characteristic signs of granule exocytosis was seen only in the presence of cytochalasin B. In the absence of cytochalasin B, human eosinophils have rarely been shown to extrude recognizable specific granules to their exterior. It is possible that the transport of granule contents by loaded vesicles, probably identical with the so-called small dense granules, is a mechanism to empty secondary granules in the absence of classical granule exocytosis (12). Morphologic signs of degranulation were paralleled by functional findings. C5a and, to a smaller extent, PAF induced a significant release of ECP, as well as EPO, only in the presence of cytochalasin B. These results agree with a previous report (31) that no release of EPO was observed in the absence of cytochalasin B upon stimulation with C5a or PAF as measured by luminol-dependent CL.

Besides the release of toxic granule proteins, the tissue-destructive power of eosinophils is determined

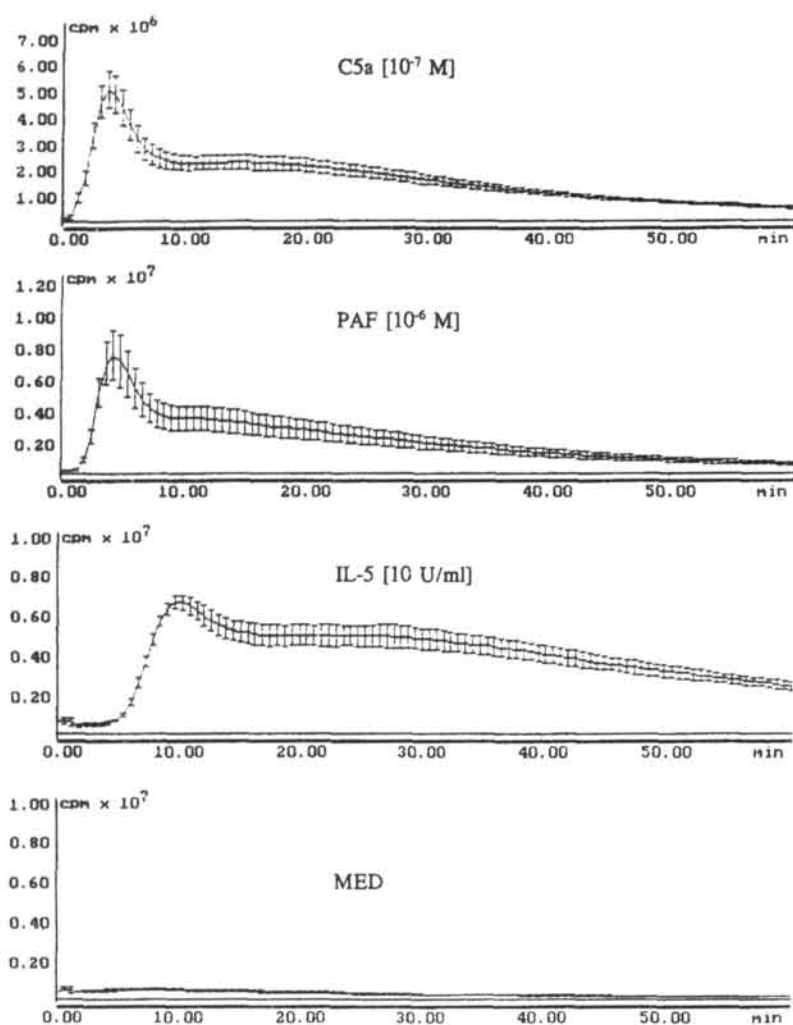


Fig. 7. Representative time course of CL response, induced by recombinant human C5a, PAF, and IL-5 in eosinophils of healthy, nonatopic blood donor. MED: background, induced by addition of medium alone. Values represent mean $\pm$ SE of six measurements on single donor. Measurements were done with Biolumat LB9505C.

by their capability to produce ROS (18). EPO catalyzes the oxidation of various targets by hydrogen peroxide in the presence of a halide, particularly bromide. Activation of this system results not only in the generation of halogenating oxidants, but also in the production of additional powerful oxygen radicals, such as singlet oxygen. Therefore, the role of C5a as activator of the oxidative burst was further investigated by lucigenin-dependent CL. On the basis of molarity, PAF was less active than C5a, which showed maximal responses at 10^{-7} – 10^{-8} M. However, maximal responses induced by both stimuli were comparable with optimal effects induced by IL-5. Eosinophil CL responses upon stimulation with C5a or PAF started immediately after addition of the stimulus and peaked at 4–6 min. In contrast, effects induced by IL-5, which was used as an additional control stimulus (27), were preceded by a short lag phase, followed by a maximum at 10–15 min. These differences may be explained in part by distinct changes of the cytoskeleton.

When the effects of C5a and PAF on eosinophils were compared with PMN of identical donors, eosinophils were found to be more responsive. Similar results were obtained when the PAF-induced super-

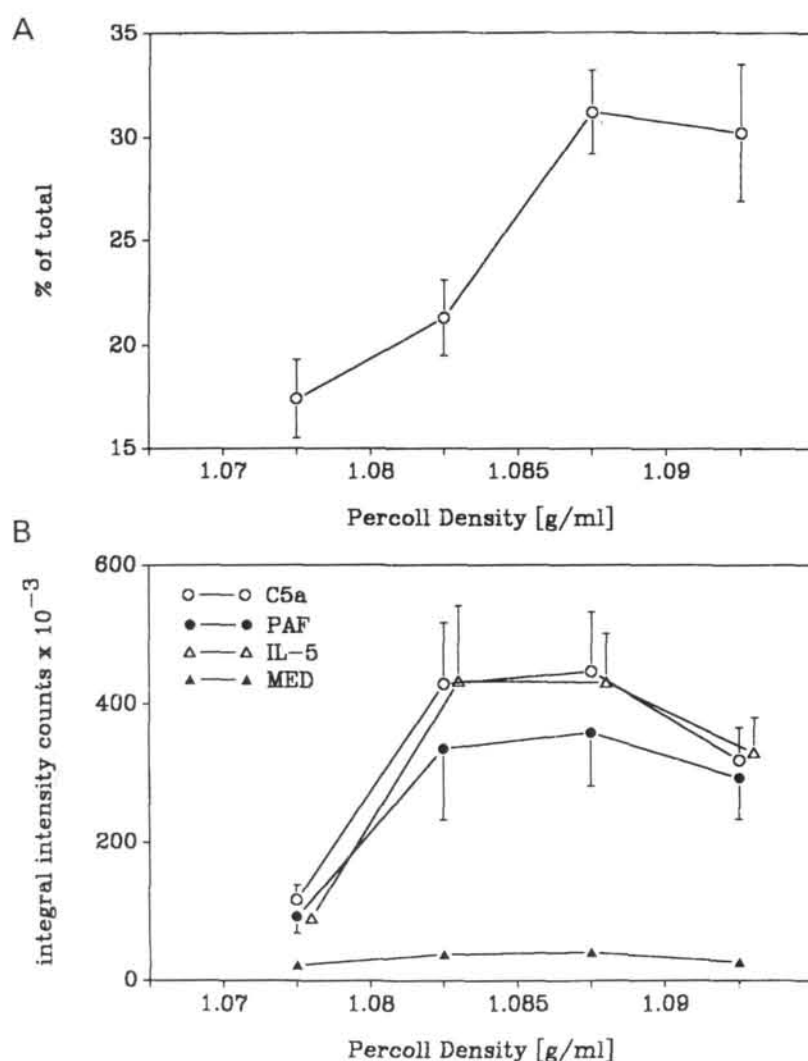


Fig. 8. Effect of C5a and PAF on hypodense and normodense eosinophil populations. After centrifugation of highly purified eosinophils on discontinuous Percoll density gradients, two hypodense and two normodense eosinophil populations were collected. A) Distribution of eosinophils on gradient indicated as percentage of total number; B) different eosinophil populations were stimulated with C5a (10^{-7} M), PAF (10^{-6} M), and IL-5 (10^2 U/ml) or medium as control (MED) for 60 min at 37°C. Results represent mean $\pm$ SEM of five experiments on five healthy, nonatopic blood donors.

oxide production was measured in patients with asthma and, particularly, in normal nonallergic subjects (62). These data clearly indicate that the differences observed between eosinophils and PMN are not due to "priming" *in vivo*, but represent an increased capacity to produce ROS upon appropriate stimulation.

The specificity of C5a-induced activation of the respiratory burst was confirmed, because the CL signal was completely blocked by a specific monoclonal antibody, whereas PAF-induced responses were not inhibited by this antibody but by a specific PAF-antagonist. These data indicate that C5a-induced CL is apparently not mediated through a simultaneous release of PAF by the eosinophils, as has been suggested for calcimycin-induced thromboxane release (17). Pretreatment of eosinophils with pertussis toxin almost completely blocked the CL responses induced by C5a and PAF. Similar results were obtained when the effect of pertussis toxin on C5a-induced release of EPO and $[Ca^{2+}]_i$ rise was

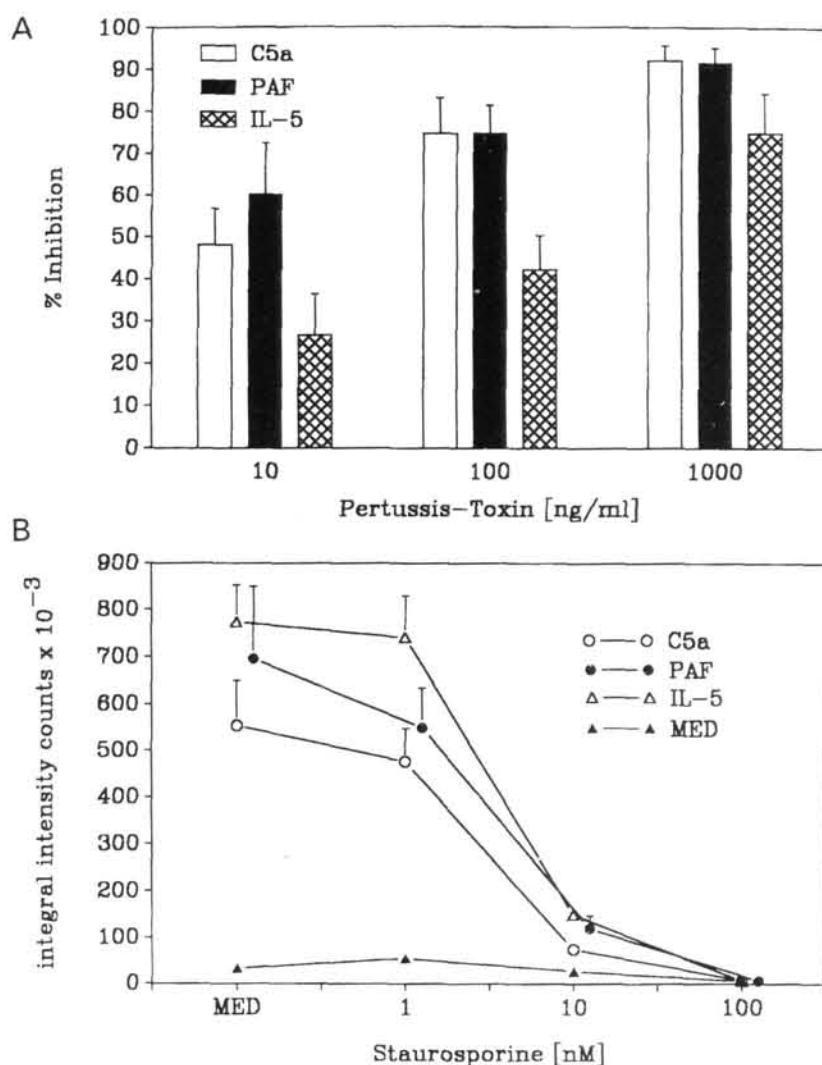


Fig. 9. Effect of pertussis toxin and staurosporine on CL response induced by recombinant human C5a, PAF, and IL-5. A) Effect of pertussis toxin: eosinophils were incubated for 90 min at 37°C in presence or absence of pertussis toxin, washed, resuspended in test buffer, and stimulated with C5a (10^{-8} M), PAF (10^{-7} M), and IL-5 (10 U/ml) for 60 min at 37°C, or remained unstimulated. Percent inhibition was calculated after subtraction of background activity observed in unstimulated cells. Results represent mean $\pm$ SEM of three experiments on three healthy, nonatopic blood donors; B) effect of staurosporine; eosinophils were preincubated with staurosporine for 2 min at 37°C before addition of C5a (10^{-7} M), PAF (10^{-6} M), and IL-5 (10^2 U/ml) or medium (MED). CL response was measured for 60 min at 37°C. Results represent mean $\pm$ SEM of four experiments on four healthy, nonatopic blood donors.

Table 2. Effect of preincubation with cytokines on eosinophil CL response

Second stimulus	C5a (10^{-7} M)	PAF (10^{-7} M)	Medium alone
Preincubation with ^{a)}	CL response (counts $\times 10^{-6}$)		
IL-2 (100 U/ml)	26.0 $\pm$ 10.5 ^{b)}	21.5 $\pm$ 6.1	0.9 $\pm$ 0.1
IL-3 (100 U/ml)	23.6 $\pm$ 4.7	14.8 $\pm$ 1.2	9.5 $\pm$ 2.5* ^{c)}
IL-5 (100 U/ml)	18.0 $\pm$ 3.2	12.5 $\pm$ 1.8	6.6 $\pm$ 0.9**
IL-8 (1 μ g/ml)	30.5 $\pm$ 11.9	14.2 $\pm$ 3.3	1.1 $\pm$ 0.1
IFN- γ (500 U/ml)	20.3 $\pm$ 7.1	19.4 $\pm$ 3.5	0.5 $\pm$ 0.07
GM-CSF (100 U/ml)	19.4 $\pm$ 4.0	16.2 $\pm$ 2.5	7.8 $\pm$ 1.4**
TNF- α (100 U/ml)	23.3 $\pm$ 6.3	16.7 $\pm$ 5.0	2.1 $\pm$ 0.3*
Medium alone	27.7 $\pm$ 9.8	22.7 $\pm$ 6.3	1.0 $\pm$ 0.2

^{a)} Eosinophils were preincubated with different cytokines or medium for 60 min at 37°C and subsequently C5a (10^{-7} M), PAF (10^{-6} M), or medium as control was added. Integral counts from 0–30-min incubation interval after addition of stimuli were measured (Biolumat LB9505C).

^{b)} Values represent mean $\pm$ SEM from six experiments on five normal, nonatopic blood donors.

^{c)} * $P \leq 0.05$; ** $P \leq 0.001$, as compared with medium alone.

suggest that the increased functional activity of these eosinophils reflects a “primed” state. In addition, it was shown that preincubation of eosinophils with GM-CSF and IL-3 significantly increased the chemotactic response to LTB₄ and f-met-leu-phe (53). Therefore, the possible “priming” effect of various cytokines supposed to influence eosinophil activity (45) on C5a- and PAF-induced eosinophil CL was investigated. However, under the conditions tested, none of these cytokines had any modulating effect. These results agree with previous reports showing that preincubation with GM-CSF, IL-3, and IL-5 did not alter C5a-induced eosinophil chemotaxis (53, 54). Moreover, C5a-induced chemotaxis was identical for allergic asthmatic patients and healthy subjects (54). In contrast, priming with GM-CSF, IL-3, or IL-5 resulted in significantly increased chemotactic responses to PAF (54). In the present study, however, no significant influence of the latter cytokines on PAF-induced activation of the respiratory burst was observed. The discrepancy may be explained by the concentrations of cytokines used for preincubation, because a priming effect was observed only at picomolar concentrations (53). Whether the priming effect observed with PAF *in vitro* may be of relevance *in vivo* has to be determined, because no significant alteration of PAF-induced eosinophil chemotaxis was observed in patients with allergic asthma after bronchoprovocation with allergen (55). As a means to obtain more insight into the possible role of C5a *in vivo*, highly purified eosinophils were further separated by discontinuous density gradients (43). By these investigations, so-called “hypodense” eosinophils (59) could be detected even in normal, nonatopic blood donors. A fraction of these hypodense cells showed significantly reduced CL responses after stimulation with

investigated (31). Moreover, staurosporin, an effective inhibitor of phospholipid/ Ca^{2+} -dependent protein kinase C (PKC) (10, 31), and wortmannin, an inhibitor of a pathway leading to the activation of phospholipase D, which is independent of Ca^{2+} influx or PKC activation (1, 44), completely abrogated the production of ROS induced by both C5a and PAF. These data indicate a role for pertussis toxin-sensitive G_i proteins in the activation of the respiratory burst in eosinophils upon stimulation with C5a and PAF. Moreover, signal transduction induced by these stimuli may involve both a PKC-dependent and a PKC-independent step.

In a recent report describing an increased luminol-dependent CL response in atopic patients after stimulation with f-met-leu-phe of PAF (33), the authors

PAF, IL-5, and, in particular, C5a. Whether these cells were "deactivated" by contact with the corresponding cytokines *in vivo* is a question that must be investigated in future studies. Alternatively, differences in expression or affinity of eosinophil-specific C5a-receptors (16) may account for the decreased responsiveness, particularly because there was a second population of hypodense cells showing responses like normodense cells.

In conclusion, the data presented in this study extend the spectrum of actions of C5a to include *de novo* production of ROS, and release of the preformed granular proteins, EPO and ECP. Since the activation of the complement system may contribute to the pathogenesis of various disorders, including asthma, anaphylaxis, and pseudoallergic reactions, compounds such as C5a derived from the complement system may play an important role in eosinophil activation during active disease. The observation that complement components and deposits are present in bronchial secretions and in the airway mucosa in lung disease (8, 20, 39, 46, 47, 49) suggests that C5a may not only stimulate circulating eosinophils but also contribute to eosinophil activation in tissue close to the inflammatory focus.

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