The Chemokine RANTES Is More than a Chemoattractant: Characterization of Its Effect on Human Eosinophil Oxidative Metabolism and Morphology in Comparison with IL-5 and GM-CSF

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Eosinophils were shown to play a major role in the allergic inflammatory process leading to the clinical symptoms of atopic dermatitis. Only selected cytokines are capable of inducing a chemotactic response in eosinophils. In particular, the chemokine RANTES was recently shown to be a potent eosinophil chemotaxin. To examine the role of RANTES in eosinophil activation, we investigated the effect of RANTES and other chemokines on morphology and oxidative metabolism of highly purified eosinophils of normal nonatopic blood donors by assessment of functional as well as morphologic criteria. RANTES, and, to a lesser extent, MIP-1α significantly induced the production of reactive oxygen species by human eosinophils, whereas MCP-1, MIP-1β, and interleukin (IL)-8/NAP-1 had no significant effects. RANTES stimulated only a subpopulation of the normal eosinophils. With the exception of IL-8, none of the cytokines tested had any significant effect on polymorphonuclear neutrophilic granulocytes. By scanning electron microscopy, RANTES induced characteristic changes that were completely abrogated in the presence of cytochalasin B. Based on functional and ultrastructural assays significant extracellular but not intracellular H_2O_2 production was detected and completely inhibited by cytochalasin B. Separation of eosinophils by discontinous density gradients revealed the existence of two hypodense eosinophil populations, one which showed significantly reduced responses upon stimulation with RANTES. RANTES-induced production of reactive oxygen species was almost completely inhibited by staurosporine, wortmannin, or pertussis toxin. Based on these data it is evident that RANTES represents a potent eosinophil-specific activator of oxidative metabolism. Besides its chemotactic activity on T cells and eosinophils, therefore, RANTES may be involved in the functional activation of eosinophils in the skin of patients with atopic dermatitis. *Key words: ultrastructure/cytokines/chemiluminescence/hydrogen peroxide. J Invest Dermatol 102:906–914, 1994*

Eosinophils are thought to be of major importance as effector cells mediating the pathogenetically relevant late-phase reaction, which is associated with significant destruction of the surrounding tissue [1]. Recent evidence indicates that these cells play an important role in the propagation of the inflammatory response in atopic diseases, particularly in bronchial asthma [2,3] and atopic dermatitis [4,5]. Immigration and local activation of eosinophils represent characteristic signs of atopic inflammation. The mechanisms of tissue-specific eosinophil recruitment and activation are not fully understood at present and involve a complex interplay of various pathways [6]. Several humoral mediators and cytokines are capable of activating the functional activity of eosinophils, e.g., C5a, platelet-activating factor, interleukin (IL)-5, and IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF). Whereas IL-5 was shown to be an eosinophil-selective chemoattractant [7] and trigger of the oxidative burst [8,9], the effect of other stimuli is not specific for eosinophils but may also trigger the metabolic activity of other leukocytes, particularly of polymorphonuclear neutrophilic granulocytes (PMNs). Very recently, a novel family of low molecular mass cytokines with distinct structural and functional homology has been defined, the RANTES/SIS cytokine family, which is identical with the C-C branch of the Platelet Factor 4/intercrine superfamily, recently renamed the "chemokine superfamily" [10]. RANTES, a member of that family originally described as a "memory" T-cell–selective attractant was shown to be a potent chemotaxin for human eosinophils, but not for human PMNs [11–13]. Chemotactic migration of cells, however, is often associated with the activation of the oxidative burst. To examine the potential role of RANTES in eosinophil activation, we investigated the effect of RANTES on morphology and oxidative metabolism of highly purified eosinophils of normal nonatopic blood donors in comparison with IL-5 and GM-CSF.

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Abbreviations: CL, chemiluminescence; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

MATERIALS AND METHODS

Cytokines and Stimuli Recombinant human RANTES, MIP-1α, and MIP-1β were a kind gift from Dr. T. Schall, Genentech, San Francisco, CA. Recombinant human MCP-1 (MCAF) was obtained from Pepro Tech, Rocky Hill, NJ. Recombinant human leukemia inhibiting factor (LIF), IL-5, GM-CSF, and monocyte-derived IL-8 were from Genzyme, Cambridge, MA. Before testing, all cytokines were diluted in phosphate-buffered saline (PBS) containing 50 μg/ml bovine serum albumin (BSA). *Escherichia*

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coli-derived recombinant human C5a was obtained from Sigma, Munich, Germany.

Reagents and Antibodies Ficoll-Paque solution was obtained from Pharmacia (Freiburg, Germany); purified BSA, lucigenin, luminol, phorbol-myristate-acetate (PMA), horse radish peroxidase type I, catalase, and cytochalasin B were obtained from Sigma. Staurosporine and pertussis-toxin were from Calbiochem-Novabiochem, Bad Soden, Germany. Wortmannin was a kind gift from Dr. D. Römer, Sandoz, Basel, Switzerland. Culture media and antibiotics were purchased from Biochrom-Seromed (Berlin, Germany). $CeCl_3$ was from Fluka, Buchs, Switzerland. Reagents and media for electron microscopy were obtained from Roth, Karlsruhe, Germany. A monoclonal mouse immunoglobulin (Ig) G2a against CD16 (BW 209/2) was a gift from Dr. R. Kurrle, Behring Werke, Marburg, Germany.

Isolation of Human Granulocytes Human granulocytes were isolated from heparin-anticoagulated venous blood of normal nonatopic blood donors as described previously [14]. 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 in a modification of a method described previously [15]. For this purpose immunomagnetic beads (Dynabeads M-450, Dynal, Norway) were coated with monoclonal αCD16. In brief, 2 ml beads (4×10^8 beads/ml) were mixed with 50 μl αCD16 (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 retrieved using a Dynal Magnetic Particle Concentrator MPC-6. αCD16-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. One milliliter of granulocytes was centrifuged in Minisorp tubes for 7 min at $820 \times g$ at 4°C, the supernatant was aspirated, and subsequently 500 μl of the αCD16-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 PMNs, which were coupled to the beads, were removed magnetically using the MPC-6 device. The 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. If necessary, the magnetic purification procedure was repeated once more. Eosinophils were quantitated with Kimura stain [16] in a Neubauer counting chamber. Cytospin preparations of the cells had a purity of > 95% as shown by Pappenheim stain.

Separation of Eosinophils on Discontinous Percoll Gradients Highly purified eosinophils were further separated by centrifugation on Percoll (Kabi Pharmacia, Freiburg, Germany) density gradients [17]. For this purpose five-step discontinous density gradients (750 μl of each interface per gradient) were formed with a peristaltic pump (Varioperpex II pump; Kabi-Pharmacia) 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 [17]. Eosinophils (5×10^6), purified as described above, were washed twice and resuspended in 750 μl Percoll solution (density of 1.07 g/ml). Percoll gradients were overlaid with these cells and centrifuged for 20 min at $1600 \times g$ 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 < 1.082 g/ml were defined as being hypodense [18]. 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 [9,19], which, in contrast to the luminol-enhanced reaction, is independent from the release of peroxidase [20], represents an extremely sensitive measure of the release of reactive oxygen species (ROS) from granulocytes. However, the CL response is not known to be strongly correlated with the production of a particular ROS. 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 [21,22]. In brief, eosinophils were suspended to a density of 5×10^4 cells/ml in HEPES-buffered HBSS, pH 7.4, containing 200 μM lucigenin and 1 mg/ml BSA. Aliquots (100 μl) containing 5×10^3 cells each were placed into flat-bottom white microtiter plates (Microfluor; Dynatech Deutschland, Denkendorf, Germany). Measurements were performed in triplicate at 37°C. Integral counts from a 0–60-min incubation interval after the addition of stimuli to the eosinophils were measured; they are indicated as integral intensity counts $\times 10^{-3}$ in Figs 1–7 and Table I.

For CL time-kinetic studies, eosinophils were suspended to a density of 2.5×10^5 cells/ml in HEPES-buffered HBSS, pH 7.4, containing 200 μM lucigenin and 1 mg/ml BSA. Aliquots (200 μl) containing 5×10^4 cells each were placed into unsealed polystyrene luminescence tubes (Berthold, Wildbad, Germany) and stored at 4°C for a maximum of 2 h prior to use. Measurements were performed at 37°C in two six-channel Biolumat LB 9505C (Berthold). Integral counts from a 0–60-min incubation interval after addition of stimuli to the granulocytes were measured, indicated as counts $\times 10^{-6}$ in Fig 3.

Extracellular and Intracellular Production of H_2O_2 Extracellular and intracellular production of H_2O_2 were determined using a modification of a sensitive chemiluminescence technique described previously [23,24]. In brief, 50 μl eosinophils at a concentration of 2×10^5/ml HBSS/BSA were distributed into flat-bottom white microtiter plates. Subsequently, 50 μl of the reaction mixtures were added and incubated with cells for 20–30 min at 37°C. Subsequently, stimuli or medium as control were added. Measurements were performed in triplicate at 37°C. Integral counts from a 0–60-min incubation interval after addition of stimuli to the eosinophils were measured and indicated as integral intensity counts $\times 10^{-3}$. The reaction mixtures for measurement of extracellular H_2O_2 production consisted of 2 mM NaN_3, 9 U/ml horseradish peroxidase (HRPO) type I, and 10^{-6} M luminol with or without the addition of 10 μg/ml cytochalasin B, whereas the cocktail used for detection of intracellular H_2O_2 production contained 4×10^3 U/ml catalase and 10^{-6} M luminol with or without the addition of 10 μg/ml cytochalasin B.

Release of Eosinophil Peroxidase (EPO) and Eosinophil Cationic Protein (ECP) For measurement of EPO and ECP release, eosinophils were suspended at a concentration of 5×10^5/ml HBSS/BSA in 96-well V-bottom tissue culture plates using 5×10^4 cells/well. After preincubation of the eosinophils for 5 min at 37°C in the presence or absence of 5 μg/ml cytochalasin B, stimuli or medium were added. Thereafter eosinophils were incubated for 30 or 120 min at 37°C, slightly shaken on a Titertek microplate shaker. Following this incubation, cells were sedimented by centrifugation for 5 min at $600 \times g$ at 4°C and supernatants were removed carefully. Subsequently, 100 μl 0.3% cetyl-trimethylammonium bromide (CETAB) was added to 100 μl supernatant each 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 μl ECL immunoassay signal reagent free of NaN_3 (Amersham, Braunschweig, Germany) was added to 10 μl supernatant in flat-bottom white microtiter plates. Measurements were performed in triplicate for 30 min at 37°C. EPO activity contained in the supernatants was expressed as intensity integral counts $\times 10^{-3}$ in Fig 7. Interassay reproducibility was tested by means of standard curves using serial dilutions of eosinophil lysates (50% to 0.5% of total content). Eosinophil lysates were generated as follows: 200 μl 0.6% CETAB was added to 100 μl eosinophils at a concentration of 5×10^5/ml, the mixture was incubated for 60 min at room temperature on a shaker, centrifuged for 5 min at $600 \times g$ at 4°C, and the supernatant was immediately frozen at −70°C. ECP concentration in the supernatants was determined by means of a commercially available double antibody radioimmunoassay (ECP-RIA; Kabi-Pharmacia) [25].

Ultrastructural Detection of Eosinophil Activation To evaluate the effect of stimuli on eosinophils by ultrastructural criteria [26,27], eosinophils were incubated at a concentration of 1×10^6/ml in microtubes for transmission and scanning electron microscopy, respectively (TEM and SEM). Thereafter, stimuli or medium were added to the eosinophils. Immediately, after addition of the stimuli, 30 μl of the mixture was transferred to Bio Rad adhesion slides (Bio Rad, Munich, Germany) and incubated for 30 min at 37°C. Thereafter, eosinophils were fixed for TEM or SEM as described below [26,27]. 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 described [9]. 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, fixed as described above, and subsequently 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 os-

Table I. Effect of Chemotactic Cytokines on Eosinophil Oxidative Metabolism

Cytokine	Concentration[a] (ng/ml)	Integral Counts × 10^{-3} [b]	N	p versus MED
RANTES	100	266.8 ± 39.6	9	0.001
MCP-1	100	35.8 ± 6.6	3	NS
MIP-1α	100	58.6 ± 13.1	5	0.01
MIP-1β	100	38.8 ± 8.5	5	NS
HILDA/LIF	100	32.1 ± 18.3	4	NS
NAP-1/IL-8	100	35.1 ± 12.2	4	NS
MED[c]		22.4 ± 3.9	9	

[a] Maximal concentration of cytokine tested.
[b] Mean ± SEM.
[c] Background induced by addition of medium alone.

mium 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 were expressed as mean ± SEM. All data were analyzed nonparametrically using the Mann-Whitney U-test. Significance was accepted for $p \leq 0.05$.

RESULTS

Effect of RANTES on the Morphology of Human Eosinophils By SEM, unstimulated eosinophils were rounded, with a ruffled surface membrane (Fig 1*A*). Upon stimulation with RANTES (100 ng/ml) only a subpopulation of the eosinophils appeared to be firmly adherent to the glass surface, exhibiting a characteristic spindle-like shape, whereas the remaining cells retained their spherical shape (Fig 1*B*). Because eosinophil preparations of 97–99% purity were used in these experiments, unstimulated cells did not represent contaminating PMNs. In contrast to stimulation with RANTES, all eosinophils treated with IL-5 (10^2 U/ml) adhered to the glass surface cells, developing long pseudopodia-like protrusions, mainly at one side of the cell (Fig 1*C*). Morphologic changes induced by GM-CSF (10^2 U/ml) were quite different; eosinophils were flattened and developed a large broad pseudopodium (Fig 1*D*). Stimulation of eosinophils in the presence of cytochalasin B, which interferes with the contractile microfilament functions [28], completely abrogated the characteristic morphologic transformation of the cells by all cytokines tested (data not shown).

By TEM, unstimulated eosinophils contained dense, specific granules displaying centrally located cristalloid cores and an outer matrix, all bounded by a typical granule membrane (Fig 1*E*). Only a few small dense, membrane-bound granules and some vesiculotubular structures were seen. Golgi vesicles, mitochondria, and other organelles appeared to be normal. After addition of RANTES to the eosinophils, only a part of the cells showed characteristic signs of activation; some secondary granules of the eosinophils lost portions or all of the normally dense matrix (see *arrow* in Fig 1*F*). In some cells unchanged dense cores remained in an otherwise empty membrane-bound granule. In addition, RANTES induced the appearance of vesicular structures in the matrix of specific granules (Fig 1*F*) and increased numbers of small electron-dense cytoplasmic granules. Many electron-lucent vesicles derived from the strongly activated Golgi apparatus were found. IL-5, however, mediated the generation of increased numbers of specific granules containing vesicular structures in the matrix, as compared to stimulation with RANTES (note *arrow* in Fig 1*G*). Like RANTES, IL-5 produced numerous small electron-dense granules as well as electron-light vesicles. Increased numbers of cytoplasmic lipid bodies, distinct from unstimulated cells, were observed upon stimulation with IL-5 (Fig 1*G*). Upon stimulation with GM-CSF, vesicular structures were present in the matrix of only a few specific granules (note *arrow* in Fig 1*H*). In contrast to stimulation with RANTES, GM-CSF produced fewer small electron-dense granules. Like IL-5, upon stimulation with GM-CSF increased numbers of cytoplasmic lipid bodies were observed (Fig 1*H*). In general, no characteristic signs of piecemeal degranulation, however, were detected with any of the stimuli tested.

Effects of RANTES on the Chemiluminescence Response of Human Eosinophils RANTES induced a dose-dependent production of reactive oxygen species (ROS) as measured by lucigenin-dependent chemiluminescence. Significant effects were observed even in the range of 10–100 ng/ml (Fig 2*A*); higher concentrations were inhibitory (data not shown). Maximal responses upon stimulation with GM-CSF or IL-5, however, were significantly higher than optimal effects induced by RANTES (Fig 2*B*). Moreover, the dose-response relation of RANTES-induced effects was focused on a small range of concentration, which was apparently different from effects induced by GM-CSF or IL-5 (Fig 2*A,B*).

The CL response induced a typical time course starting immediately after addition of the stimulus, with a peak at about 4–6 min, followed by an increase of baseline activation of the eosinophils for about 1 h (Fig 3). In contrast, CL response upon stimulation with IL-5 and GM-CSF 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 baseline activity.

In addition to RANTES, other members of the C-C branch of chemokines were tested. Only MIP-1α was shown to induce a small but significant chemiluminescence response in human eosinophils in the range of 10–100 ng/ml (Fig 2*A*, Table I). The structurally related MIP-1β as well as MCP-1 were inactive. Similar results were obtained for IL-8 and LIF/HILDA [29,30].

Effects of RANTES on the Chemiluminescence Response of Human PMN In contrast to IL-8 or GM-CSF, which were able to induce a significant CL response in human PMNs, MIP-1α, MIP-1β, and MCP-1 had no measurable effect on PMNs (data not shown). RANTES and IL-5, however, induced small but statistically significant CL responses in PMN preparations that were contaminated with 4–6% eosinophils (data not shown). Therefore, these contaminating eosinophils could be responsible for the effects observed. Alternatively, CL responses might be due to the interaction of PMNs with activated eosinophils, because PMNs in mixed granulocyte preparations, particularly in atopic patients, are activated indirectly by factors released from IL-5–activated eosinophils or by an IL-5–mediated, adherence-dependent mechanism [9].

Effect of Inhibitors on the Chemiluminescence Response of Human Eosinophils Preincubation of eosinophils with 5 μg/ml cytochalasin B for 5 min before addition of RANTES (100 ng/ml), IL-5 (10^2 U/ml), or GM-CSF (10^2 U/ml) completely inhibited the chemiluminescence response (data not shown). In contrast, there was only a partial inhibition when PMA (10 ng/ml) was used as stimulus (3264 ± 715 versus 985 ± 85 counts × 10^{-3}; mean ± SEM of six blood donors). Pretreatment of eosinophils with 100 ng/ml pertussis toxin for 120 min at 37°C [31] resulted in an almost complete inhibition (85 ± 11%) of chemiluminescence responses induced by RANTES (Fig 4*A*), whereas production of reactive oxygen species induced by IL-5 or GM-CSF was strongly suppressed only at higher concentrations of pertussis toxin (Fig 4*B*). In

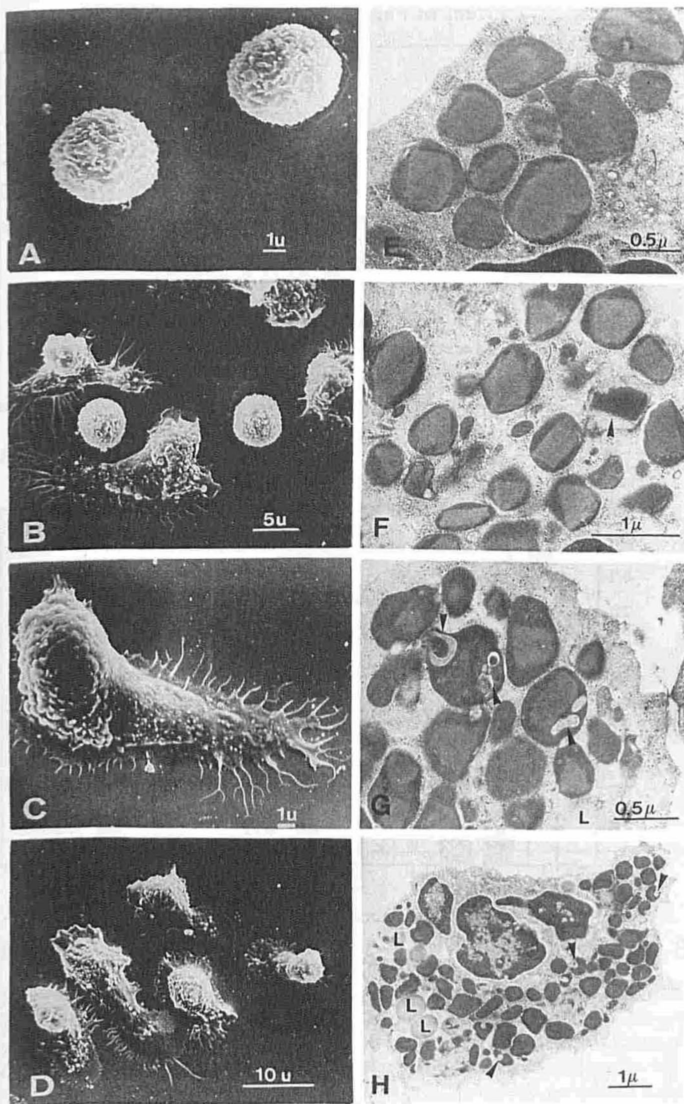
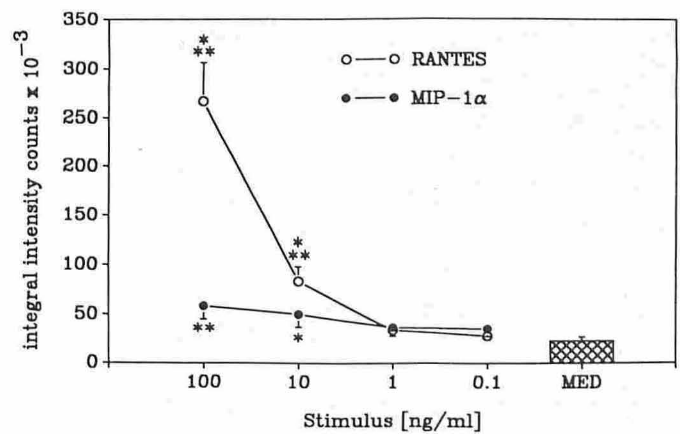


Figure 1. Effect of RANTES, IL-5, and GM-CSF on the morphology of human eosinophils. Ultrastructural detection of eosinophil activation induced by recombinant human RANTES, IL-5, and GM-CSF. *A)* For unstimulated eosinophils, medium was added instead of stimuli, 30 min at 37°C, SEM; *B)* eosinophils stimulated with RANTES (100 ng/ml), 30 min at 37°C, SEM; *C)* eosinophils stimulated with IL-5 (10^2 U/ml), 30 min at 37°C, SEM; *D)* eosinophils stimulated with GM-CSF (10^2 U/ml), 30 min at 37°C, SEM; *E)* for unstimulated eosinophils, medium was added instead of stimuli, 30 min at 37°C, TEM; *F)* eosinophils stimulated with RANTES (100 ng/ml), 30 min at 37°C, TEM; *G)* eosinophils stimulated with IL-5 (10^2 U/ml), 30 min at 37°C, TEM; *H)* eosinophils stimulated with GM-CSF (10^2 U/ml), 30 min at 37°C, TEM. Eosinophils used in these experiments had a purity of 98–99%. The experiment is representative of six essentially identical experiments on eosinophils of healthy nonatopic blood donors. L, cytoplasmic lipid bodies.



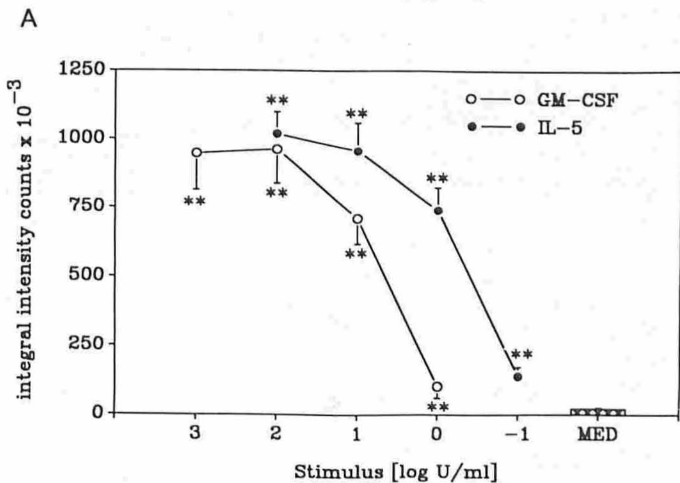


Figure 2. Effects of RANTES, IL-5, and GM-CSF on the oxidative metabolism of human eosinophils. Chemiluminescence responses of purified eosinophils from healthy nonatopic blood donors are shown. Effect of recombinant human *(A)* RANTES, MIP-1α, *(B)* GM-CSF, and IL-5. Integral counts (60-min) were measured using a Hamamatsu MTP reader. Values represent mean ± SEM of experiments on five to nine healthy nonatopic blood donors. MED, background, induced by addition of medium alone. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus background.

contrast, PMA-induced CL was only partially inhibited even at a concentration of pertussis toxin of 1 μg/ml. Pretreatment of the eosinophils with staurosporine [32] inhibited the chemiluminescence responses upon stimulation with RANTES, IL-5, or GM-CSF in dose-dependent fashion. Complete inhibition was observed at a concentration of 100 nM (Fig 4*C*). Pretreatment of the eosinophils with 1 μM wortmannin [33,34] totally abrogated the responses induced by RANTES, IL-5, or GM-CSF (data not shown).

Effect of RANTES on the Chemiluminescence of Hypodense and Normodense Eosinophils Separation of eosinophils on discontinous Percoll gradients revealed at least two hypodense cell populations that were collected at the interphase between 1.07/1.08 g/ml and 1.08/1.085 g/ml (Fig 5*A*). It was noted that 17.4 ± 1.9% of the cells belonged to the population with the lowest density, which was significantly resistant to stimulation with RANTES, IL-5, or GM-CSF, in comparison to the normodense cell populations (Fig 5*B*). In contrast, there were no significant differences in the chemiluminescence response of the second hypodense cell population (21.3 ± 1.8% of the eosinophils), as compared with normodense eosinophils (Fig 5*B*).

Effect of RANTES on Intracellular and Extracellular Production of H_2O_2 Based on functional assay systems, a significant extracellular H_2O_2 production was detected upon stimulation with RANTES (100 ng/ml), IL-5 (10^2 U/ml), or GM-CSF (10^2 U/ml) (105 ± 29, 496 ± 116, 344 ± 55, and 13 ± 3 counts $\times 10^{-3}$ for IL-5 and GM-CSF induced a significant intracellular production of H_2O_2, no significant responses were detected in RANTES-stimulated eosinophils (4.6 ± 0.9, 36.5 ± 9.1, 58.2 ± 19.8, and 3.3 ± 0.3 integral intensity counts $\times 10^{-3}$ for RANTES, IL-5, GM-CSF, and medium, respectively). Both intracellular as well as extracellular production of H_2O_2 were blocked completely in the presence of cytochalasin B (data not shown). Identical results were obtained when hydrogen peroxide production was visualized by an ultrastructural technique. Upon stimulation with RANTES, the reaction product was observed only on the outer surface of the plasma

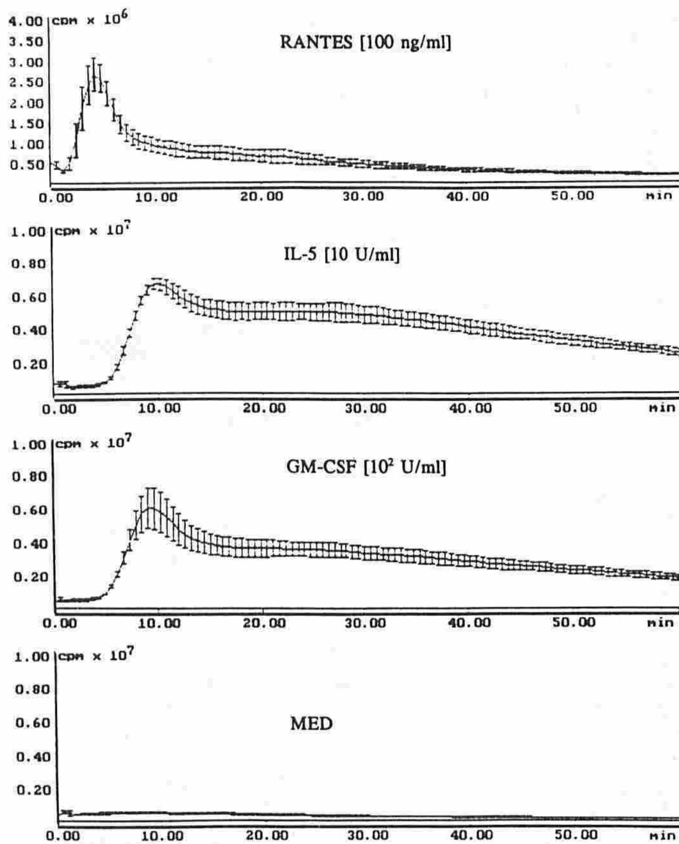


Figure 3. Representative time course of the chemiluminescence response upon stimulation with recombinant human RANTES, IL-5, and GM-CSF in human eosinophils. MED, background, induced by addition of medium alone. Values represent the mean ± SD of six measurements on a single healthy nonatopic blood donor. Measurements were done using a Biolumat LB9505C.

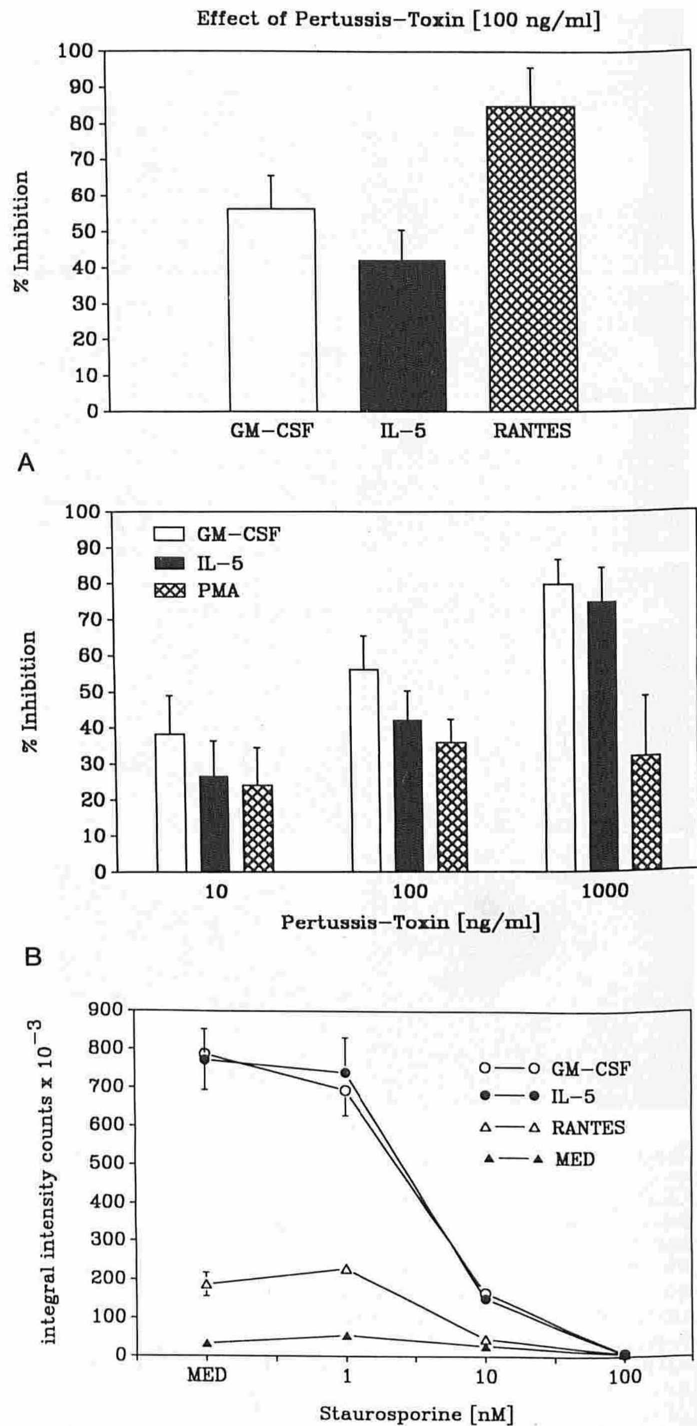


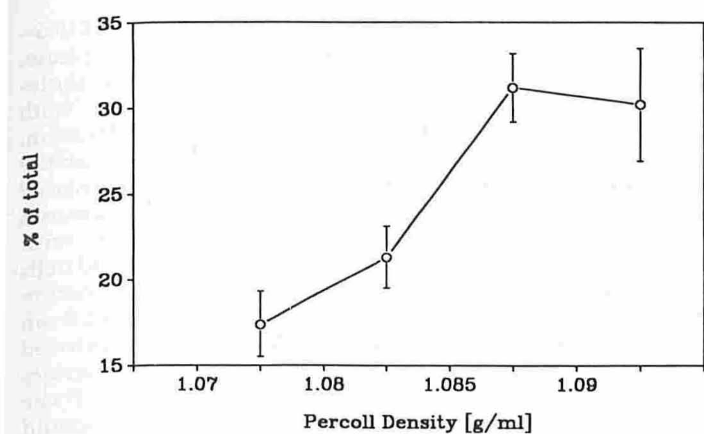
Figure 4. Effect of pertussis toxin and staurosporine on the chemiluminescence response of human eosinophils induced by recombinant human RANTES, IL-5, and GM-CSF. *A)* Effect of pertussis toxin on RANTES-induced CL response. Eosinophils were incubated for 90 min at 37°C in the presence or absence of pertussis toxin, washed, resuspended in test buffer, and stimulated with RANTES (100 ng/ml), IL-5 (10 U/ml), and GM-CSF (10 U/ml) for 60 min at 37°C or remained unstimulated. Percent inhibition was calculated after subtraction of the background activity observed in unstimulated cells. The results represent the mean ± SEM of three experiments on three healthy nonatopic blood donors. *B)* Dose-related inhibition of CL response induced by IL-5 (10 U/ml), GM-CSF (10 U/ml), or PMA (10 ng/ml). *C)* Effect of staurosporine. Eosinophils were preincubated with staurosporine for 2 min at 37°C before addition of RANTES (100 ng/ml), IL-5 (10^2 U/ml), GM-CSF (10^2 U/ml), or medium (MED). Chemiluminescence response was measured for 60 min at 37°C. Results represent the mean ± SEM of four experiments on four healthy nonatopic blood donors.

membrane (Fig 6*A*). Hydrogen peroxide production appeared to be RANTES, IL-5, GM-CSF, and medium, respectively). Whereas "focused" on distinct parts of the plasma membrane. In contrast, there were no signs of intracellular production of H_2O_2. Upon stimulation with IL-5 or GM-CSF, however, significant amounts of the reaction product were observed at the outer surface of the plasma membrane, mainly between adjacent cells, and particularly at the end of the pseudopodia, but also at the luminal part of the small intracytoplasmic vacuoles (Fig 6*B,C*). Extracellular as well as intracellular production of ROS was completely inhibited by cytochalasin B (data not shown).

Effect of RANTES on the Release of ECP and EPO In contrast to its effect on eosinophil oxidative metabolism, no significant exocytosis of EPO and ECP (Fig 7) in the supernatant could be detected even at highest concentrations of RANTES. Similar results were obtained for MIP-1α, IL-5, or GM-CSF (data not shown). Significant release of both proteins was observed only upon stimulation with C5a in presence of cytochalasin B. Increase of the incubation time from 60 to 120 min did not induce any significant change in the results (Fig 7).

DISCUSSION

In addition to its chemotactic activity, RANTES was shown to be a potent activator of the oxidative burst in eosinophils in this study. Production of reactive oxygen species associated with the release of toxic granule proteins determines the tissue-destructive power of eosinophils [1]. Eosinophil peroxidase, as a constituent of eosinophil granules, catalyzes the oxidation of various targets by hydrogen peroxide in the presence of halide, particularly bromide [35]. Activation of this system results not only in the generation of halogenating oxidants, but also in the production of additional powerful



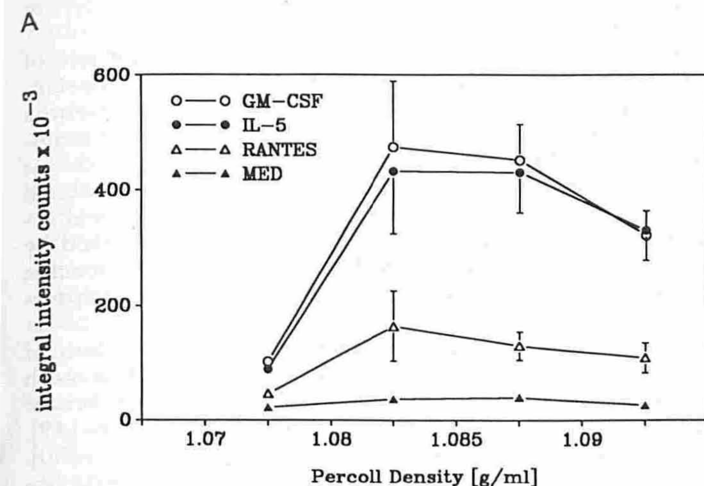
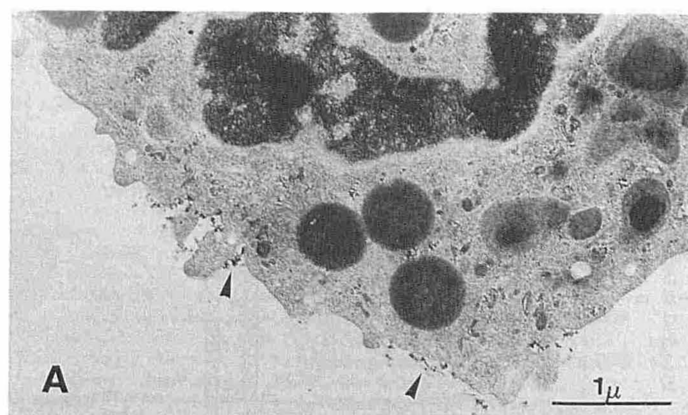
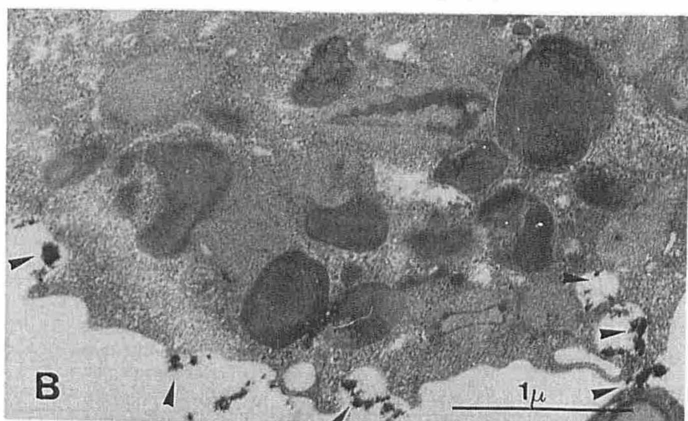


Figure 5. Effect of RANTES, IL-5, and GM-CSF on the oxidative burst of hypodense and normodense eosinophil populations. Following centrifugation of highly purified eosinophils on discontinous Percoll density gradients two hypodense and two normodense eosinophil populations were collected. *A)* Distribution of eosinophils on the gradient indicated as percent of total number; *B)* The different eosinophil populations were stimulated with RANTES (100 ng/ml), IL-5 (10^2 U/ml), and GM-CSF (10^2 U/ml), or medium as a control (MED), and the chemiluminescence response was measured for 60 min at 37°C. Results represent the mean ± SEM of five experiments on five healthy nonatopic blood donors.





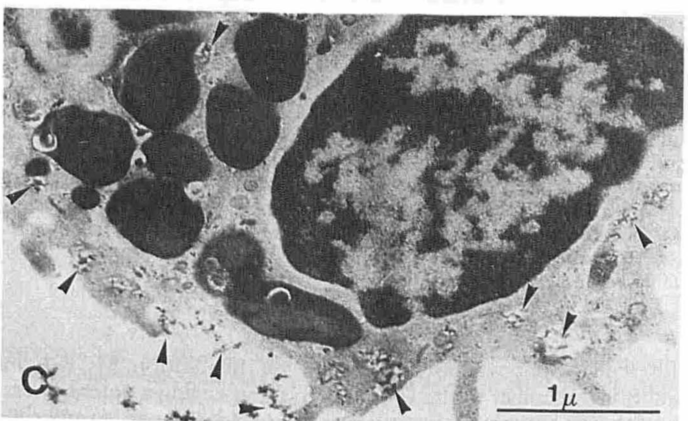
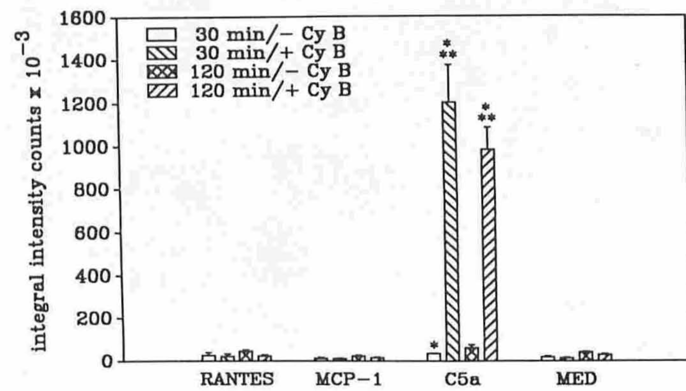


Figure 6. Ultrastructural detection of H_2O_2 production by human eosinophils by recombinant human RANTES, IL-5 and GM-CSF. *A)* Eosinophils of a healthy nonatopic blood donor stimulated with RANTES (100 ng/ml), 30 min at 37°C, ultrastructural detection of hydrogen peroxide; *B)* eosinophils stimulated with IL-5 (10^2 U/ml), 30 min at 37°C, ultrastructural detection of hydrogen peroxide; *C)* eosinophils stimulated with GM-CSF [10^2 U/ml], 30 min at 37°C, ultrastructural detection of hydrogen peroxide. *Arrows*, production of hydrogen peroxide is visualized by the detection of the electron-dense precipitate of $Ce(OH)_3OOH$ (for details see *Materials and Methods*). The experiment is representative for five essentially identical experiments on healthy nonatopic blood-donors.

oxygen radicals, such as singlet oxygen [36]. Release of these toxic agents by eosinophils during allergic inflammation [2–5] is now considered to be a major pathogenic step in the pathophysiology of atopic diseases.

Lucigenin-dependent CL represents a highly sensitive measure for the production of ROS, particularly for the release of superoxide anion, because ≥70% of the signal could be blocked by superoxide dismutase [19]. Therefore, this technique was used to investigate the effect of RANTES on human eosinophils. Based on this assay system, maximal responses induced by RANTES were only 30% of optimal effects induced by IL-5 or GM-CSF. Significant CL responses were only seen in a very small dose range, from 10 to 100 ng/ml. However, there may be remarkable differences between natural and recombinant human chemokines, proabaly due to variations in the tertiary structure of the proteins (J. Schröder, personal communication).

When other members of the chemokine family were tested, only MIP-1α, which was recently shown to be an activator of basophils and mast cells [37], induced a small but statistically significant CL response in eosinophils. This is in contrast to a previous report [12] showing the MIP-1α induced a chemotactic response in eosinophils but no production of hydrogen peroxide in a luminol-dependent CL system. This may be explained by the lack of sensitivity of that method as compared to the highly sensitive photon-imaging system used in our study [21]. Nevertheless, MIP-1β, which is more homologous to MIP-1α than is MIP-1α to RANTES [10], did not influence eosinophil chemotaxis [12] and the release of ROS. The discrepancy between homology and biologic activity of these related molecules is further supported by their capability to attract



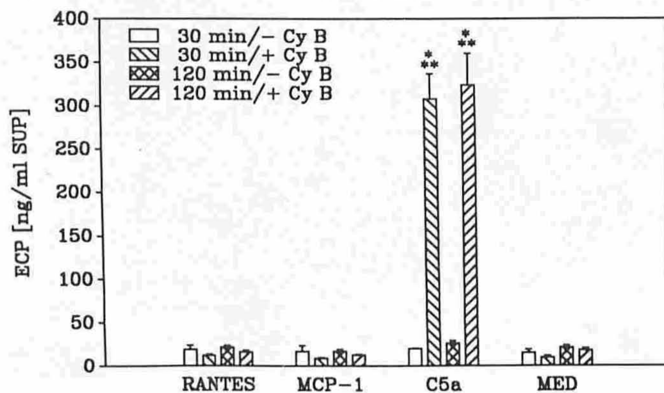


Figure 7. Release of EPO and ECP by eosinophils of healthy nonatopic blood-donors upon stimulation with RANTES, MCP-1, and C5a. Eosinophils were stimulated with recombinant human RANTES (100 ng/ml) and MCP-1 (100 ng/ml) in the presence or absence of cytochalasin B (5 μg/ml) for 60 or 120 min at 37°C. Recombinant human C5a (10^{-7} M) was used as a positive control. EPO activity was measured in supernatants using a highly sensitive enhanced luminescence assay. ECP concentration was determined by means of a double-antibody radioimmunoassay. MED, spontaneous release after addition of medium. Values represent the mean ± SEM of triplicate experiments on four to five healthy nonatopic blood donors. ***$p \leq 0.001$ versus MED.

different lymphocyte populations [38,39]. In addition, MCP-1, an additional member of the C-C branch representing a potent direct activator of basophil degranulation [40,41], did not show any significant effect on eosinophil chemotaxis [42] and respiratory burst. These differences may be partially explained by the observation that MIP-1α, MIP-1β, and MCP-1, and RANTES bind to a common receptor but with varying affinities [43]. The RANTES/MIP-1α receptor was recently shown to belong to the G-protein–coupled receptor superfamily [44]. Our data clearly establish a role for pertussis toxin-sensitive G_i proteins in the activation of the respiratory burst in eosinophils upon stimulation with RANTES, as well as with IL-5 or GM-CSF. Moreover, because staurosporine, an effective inhibitor of phospholipid/Ca^{++}-dependent proteinkinase C (PKC) [31,32], and wortmannin, an inhibitor of a pathway leading to the activation of phospholipase D that is independent from Ca^{++} influx or PKC activation [33,34], completely abrogated the production of reactive oxygen species induced by all stimuli tested, signal transduction may involve both a PKC-dependent and -independent step.

Like IL-5, RANTES was proven to be an eosinophil-selective stimulus of eosinophil oxidative metabolism whereas GM-CSF is able to activate both eosinophils and PMNs [9,26]. Eosinophil CL responses upon stimulation with RANTES were comparable to the effect of classical eosinophil chemotaxins, such as C5a or PAF [45], starting immediately after addition of the stimulus, whereas IL-5– or GM-CSF–induced effects were preceded by a short lag phase. These differences may be due to distinct changes of the cytoskeleton, which will be discussed below. Upon stimulation with RANTES only a part of the eosinophils showed signs of activation. This may partially be explained by our finding that a part of the "hypodense" eosinophils [18], which were detected even in normal nonatopic blood donors, showed significantly reduced CL responses following stimulation with IL-5, GM-CSF, and, particularly, with RANTES. At present, it can only be speculated whether these cells are "deactivated" to specific stimulation as a result of preactivation with RANTES *in vivo* or during isolation by RANTES derived from platelets [11]. Because another part of the hypodense cells showed responses that were not different from normodense cells, changes in susceptibility are possibly due to different expression of the RANTES/MIP-1α receptor. However, the latter hypothesis could also explain interindividual differences of susceptibility to RANTES (data not shown).

In contrast to RANTES, the three-dimensional appearance of eosinophils upon stimulation with IL-5 or GM-CSF was different. Polarization, particularly induced by IL-5 [46], usually represents a characteristic feature of inflammatory cells responding to chemotactic factors [47]. Because all cytokines tested are chemotactic for eosinophils [48], the different cell shape of RANTES-stimulated eosinophils cannot be explained simply by different functional activities. All morphologic changes, however, could be blocked by cytochalasin B [28], supporting the concept of stimulus-specific alterations of the cytoskeleton, which may be restricted to receptor-specific transductional events.

Based on TEM, morphologic changes, which resembled those of activated tissue eosinophils, were noted upon stimulation with RANTES, GM-CSF, or IL-5. These included decreased numbers of specific granules, increased numbers of small dense granules [49], and increased numbers of membrane-bound large lipid bodies [50]. Furthermore, large amounts of elongated smooth tubules were observed in the cytoplasma of these eosinophils. Moreover, increased numbers of so-called micrograpnules, as a part of the intracytoplasmic tubulo-vesicular structure representing a unique finding for eosinophils [51], were found regularly upon stimulation with RANTES. Ultrastructural alterations of secondary granules, particularly the loss of matrix and the appearance of light vesicles, were pronounced in RANTES-stimulated eosinophils, but could also be seen with IL-5 and GM-CSF.

IL-5 and, to a lesser degree, GM-CSF, regularly induced the formation of tubulo-vesicular structures in the matrix of specific granules. These structures are thought to represent integral components of a specific granule matrix that are masked by the packed enzymic contents and only disclosed during granule lysis [52]. Most of these characteristic findings have been noted previously in normal human eosinophils undergoing "exocytosis" [53,54]. Based on functional and immunologic assay systems, however, no significant release of EPO and ECP could be detected upon stimulation with RANTES, IL-5, or GM-CSF. Comparable results have been published recently for IL-5 and GM-CSF [55]. In contrast, there are two reports describing a RANTES-induced release of ECP [12,13]. In the first paper, the amount of ECP released in the absence of cytochalasin B was comparable to stimulation with PAF (10^{-7} M). In the second report, RANTES stimulated the release of ECP only in the presence of cytochalasin B, with optimal effects corresponding to 25% of C5a (10^{-7} M). In contrast to a significant release of both ECP and EPO with C5a in the presence of cytochalasin B [31], we were unable to reproduce these findings with RANTES. In our experiments, however, eosinophils were tested at a lower cell density (10^4 cells/well) to avoid contact activation, because activated cells may also disrupt during centrifugation.

Based on ultrastructural findings, human eosinophils have rarely been shown to extrude recognizable specific granules to the cells' exterior upon cytokine stimulation. However, eosinophils cultured in the presence of IL-5 were found to lose all secondary granules [56]. It is possible that the transport of granule contents by loaded

vesicles, probably identical with the so-called small dense granules, represents a mechanism of emptying secondary granules in the absence of classical granule exocytosis. Therefore, the term "piecemeal degranulation" was coined to describe that type of release reaction. RANTES, particularly, stimulated the formation of small-sized dense granules that were originally thought to reflect the synthesis of immature new granules as a consequence of initiation of the secretory process [53]. However, at present only mast cells were shown to "regranulate" following degranulation, as shown by their synthesis of new secretory material and the function of mature cytoplasmic granules [57]. Ultrastructural findings obtained with IL-5, GM-CSF, and, particularly, with RANTES, therefore, strongly suggest intracellular mobilization or translocation of toxic granule proteins. Therefore, a localized "piecemeal degranulation" may contribute to the changes of eosinophil density observed upon stimulation with RANTES [13] and IL-5 [58]. This hypothesis was supported further by the demonstration of a localized production of hydrogen peroxide by cytokine-stimulated eosinophils, which was completely blocked in the presence of cytochalasin B. These data strongly support the concept that the production of ROS induced by these cytokines is dependent on the adherence of the cells to an appropriate surface [59].

Based on these findings it is evident that RANTES represents a potent eosinophil-specific activator of the oxidative metabolism in these proinflammatory effector cells. In addition to its chemotactic activity on T cells and eosinophils, therefore, RANTES may be an attractive candidate responsible for the selective activation of eosinophils in the skin of patients with atopic dermatitis, triggering their toxic capacities.

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