We report that responses of normal human eosinophils toward the chemokines RANTES and interleukin-8 (IL-8) are modulated and upregulated by priming with IL-5. In a modified Boyden chamber assay, we studied migratory responses toward the members of the chemokine family RANTES, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) (C-C subfamily), and IL-8, platelet factor-4 (PF-4), and neutrophil-activating peptide-2 (NAP-2) (C-X-C subfamily). These chemokines were also studied in terms of actin polymerization and ([Ca2+]i)-mobilizing properties, intracellular signals that are thought to play a role during migratory responses. We found that eosinophils showed significant migratory responses toward RANTES and IL-8 at concentrations of 10^{-8} to 10^{-7} mol/L only after priming with IL-5 (10 pmol/L). At these concentrations, PF-4, NAP-2, MCP-1, and MIP-1α induced no significant migratory responses after priming. Unprimed eosinophils only showed a significant migratory response toward RANTES (10^{-6} mol/L). Changes in [Ca2+]i were found after addition of RANTES, MIP-1α, and NAP-2 (10 nmol/L) to unprimed eosinophils. RANTES (10^{-6} to 10^{-7} mol/L) significantly induced actin polymerization both in primed and unprimed eosinophils, whereas IL-8 (10^{-6} to 10^{-7} mol/L) and MIP-1α (10^{-6} mol/L) only induced actin polymerization after priming with IL-5. NAP-2, PF-4, and MCP-1 did not affect actin polymerization. These findings are further evidence for the hypothesis that cytokines like IL-5 and locally secreted chemokines like RANTES and IL-8 are both at the basis of specific eosinophil influx into the allergic inflammatory locus.

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l-α-lysophosphatidylcholin (type I, egg yolk) were purchased from Sigma (St Louis, MO). Indo-1/AM and NBD-phallacidin (200 µM in methanol) were purchased from Molecular Probes (Junction City, OR). Ficol-paque and Percoll were obtained from Pharmacia (Uppsala, Sweden). All other materials were reagent grade. Experiments were performed in incubation buffer containing 132 mM/L NaCl, 6.0 mM/L KCl, 1.0 mM/L CaCl₂, 1.0 mM/L MgSO₄, 1.2 mM/L KH₂PO₄, 20 mM/L HEPES, 5 mM/L glucose, and 1.0% human serum albumin (HSA) (wt/vol).

Cytokines. Recombinant human (rh) IL-5 was a kind gift from Dr D. Fattah, GLAXO (Greenford, England). rhRANTES, rhIL-8 (endothelial cell-derived), rhMCP-1, and rhMIP-1α were obtained from Genzyme (Cambridge, MA). Another batch of rhRANTES was obtained from PeproTech (Rocky Hill, NJ). PF-4 was purchased from ICN Biochemicals (Cleveland, OH). NAP-2 was a gift from Dr I.J. Lindley, Sandoz Research Institute (Vienna, Austria).

Stock solutions (small samples) of all cytokines were prepared in a phosphate-buffered saline (PBS) solution, supplemented with 0.2% purified HSA, and stored at −80°C until use.

Cell isolation. Blood was obtained from healthy, normal volunteers from the Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy coat of 500 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) as described previously. To reduce the number of neutrophils in this mixed granulocyte population, the cells were subjected to discontinuous Percoll gradient (1.084/1.0) centrifugation (20 minutes, 1000 × g, room temperature). Eosinophils were subsequently isolated using the method described by Hansel et al. This isolation method is based on the fact that, in marked contrast to neutrophils, eosinophils lack the epitope on FcyRIII recognized by the monoclonal antibody CLB-FcR-gran 1 directed against CD16. As a result, highly purified eosinophils can be isolated by removing neutrophils coated with CLB-FcR-gran 1 with immunomagnetic dynabeads (Dynal, Oslo, Norway). Briefly, neutrophils were coated with a monoclonal antibody against CD16 (CLB-FcR-gran 1, 2 μg/10⁷ cells/mL) for 20 minutes at 4°C. The cells were washed twice and subsequently incubated head over head in a rotator with dynabeads at ratio of 1:2 (cells/beads) for 20 minutes at 4°C. Neutrophils were subsequently removed by a magnetic particle concentrator (MPC™, Dynal). Eosinophil purity was always more than 95% and viability was more than 98%.

Migration assay. Eosinophil migration was measured by a modification of the method of Boyden, using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). Chemotaxins or incubation buffer (30 µL) were added to the lower compartments. Two filters were placed between the lower and upper compartments. The lower filters had a pore width of 0.45 µm (Millipore, Bedford, MA; type HA) and the upper filter (cellulose nitrate) a pore width of 8 µm (thickness, 150 µm; Sartorius, Göttingen, Germany; type SM 113). Before use, the filters were soaked in incubation buffer. Purified eosinophils were placed in the upper compartments (25 µL of 5 × 10⁶ cells/mL). For IL-5 priming, cells were incubated with 10⁻¹¹ mol/L IL-5 at 37°C for 30 minutes, washed, and resuspended in incubation buffer. Unprimed control cells were also placed at 37°C for 30 minutes, washed, and resuspended in incubation buffer. The chambers were subsequently incubated for 2.5 hours at 37°C. The upper filters were removed, fixed in butanol:ethanol (20:80%), sol vol for 10 minutes, and stained with Weigert solution (composition, 1% [vol/vol] hematoxylin in ethanol mixed with a 70 mM/L acidic FeCl₃ solution at a 1:1 ratio). The filters were dehydrated with ethanol, made transparent with xylene, and fixed upside down. The number of cells per 10 high-power fields (HPFs) was determined by light microscopy (magnification, 400 ×). In this way, the number of cells (the leading front) that passed the upper filter (and migrated 150 µm) was determined.

Effect of chemokines on migratory responses of normal human eosinophils. We have tested migratory responsiveness of eosinophils derived from normal donors toward the chemokines IL-8, NAP-2, PF-4, RANTES, MCP-1, and MIP-1α. Figure 1 shows the effect of the C-X-C chemokines PF-4, NAP-2, and IL-8 in dose ranges of 10⁻¹⁴ to 10⁻⁷ mol/L in inducing eosinophil migration. In the unprimed state, none of these chemokines was able to induce a significant migratory response. However, after priming with 10⁻¹¹ mol/L IL-5 for 30 minutes at 37°C, washing, and resuspending the cells in incubation buffer, IL-8 was able to induce a significant migratory response at a concentration range of 10⁻⁸ to 10⁻⁶ mol/L, confirming our earlier studies. Figure 2 shows the effects of the C-C chemokines MIP-1α, MCP-1, and RANTES in dose ranges of 10⁻¹¹ to 10⁻⁷ mol/L in inducing eosinophil migration. Again, none of these chemokines induced a migratory response in unprimed eosinophils at these concentrations. However, RANTES was...
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Fig 1. C-C chemokine-induced migration of unprimed (○) and IL-5 (10^{-11} mol/L, 30 minutes, 37°C)-primed (●) human eosinophils. Dose ranges of 10^{-11} to 10^{-7} mol/L PF-4 (A), NAP-2 (B), and IL-8 (C) were tested. B, response to incubation buffer. Mean values ± SEM are presented of six experiments with unprimed cells and 10 experiments with IL-5-primed cells. *Significant difference from buffer control at P < .05.

able to induce a significant migratory response at a concentration range of 10^{-9} to 10^{-7} mol/L after the cells were primed with IL-5. Two batches of RANTES obtained from different companies (see Methods) were tested, but no differences were found. Both Fig 1 and Fig 2 show that priming by IL-5 results in an increased random migration (chemokinase) as shown by enhanced responses toward incubation buffer.

Table 1 shows that RANTES- and IL-8-induced migratory responses were not due to the aforementioned chemokinase activation. When optimal concentrations (10^{-7} mol/L) of these chemokines were added to the upper chamber with the IL-5-primed cells, and either incubation buffer or the same concentration of the chemokine was placed in the lower chamber, it is shown that IL-8 and RANTES are true chemotaxins for IL-5-primed eosinophils.

In another set of experiments, we tested the possibility that migratory responses of normal human eosinophils toward RANTES, MIP-1α, and IL-8 were induced at a higher concentration (10^{-6} mol/L). At this concentration, in marked contrast to IL-8 and MIP-1α, RANTES induced a significant migratory response in unprimed eosinophils (119.7 ± 9.7, n = 6; Table 2), whereas 10^{-7} mol/L in these experiments was not active (not shown). After priming with IL-5, RANTES and IL-8 induced significant migratory responses at 10^{-6} mol/L (Table 2), although these responses were not significantly different from the responses at 10^{-7} mol/L shown in Figs 1 and 2.

Chemokine-induced changes in [Ca^{2+}]_{i} in eosinophils. Chemotactants such as PAF and C5a are able to induce a rapid and transient increase in [Ca^{2+}]_{i} in eosinophils. Since this signal is one of the first events in the signal transduction cascade after agonist-receptor binding, we tried to correlate migratory responses of the chemokines with the elevation in [Ca^{2+}]_{i}. In these experiments, 1 μmol/L PAF was used as positive control. As is shown in Fig 3, from all tested chemokines, only RANTES, MIP-1α, and NAP-2 clearly induced elevations in [Ca^{2+}]_{i} in unprimed cells. Fig 3A and B show the responses toward MIP-1α, RANTES, and IL-8. Subsequent addition of a different C-C chemokine resulted in a reduced response. The sequence of addition of the different chemokines is arbitrarily chosen. Fig 3C shows

Fig 2. C-C chemokine-induced migration of unprimed (○) and IL-5 (10^{-11} mol/L, 30 minutes, 37°C)-primed (●) human eosinophils. Dose ranges of 10^{-11} to 10^{-7} mol/L MIP-1α (A), MCP-1 (B), and RANTES (C) were tested. B, response to incubation buffer. Mean values ± SEM are presented of six experiments with unprimed cells and 10 experiments with IL-5-primed cells. *Significant difference from buffer control at P < .05.
Table 1. Chemotaxis and Random Migration in Response to RANTES and IL-8

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Buffer</th>
<th>Ranties</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lower Compartment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>61.0 ± 4.2</td>
<td>152.0 ± 8.8*</td>
<td>122.0 ± 6.8*</td>
</tr>
<tr>
<td>Ranties</td>
<td>48.0 ± 4.1</td>
<td>59.0 ± 5.1</td>
<td>ND</td>
</tr>
<tr>
<td>IL-8</td>
<td>53.2 ± 2.9</td>
<td>ND</td>
<td>66.0 ± 2.6</td>
</tr>
</tbody>
</table>

RANTES and IL-8, both $10^{-7}$ mol/L, were placed only in the lower compartment of the Boyden chamber (normal migration assay), only in the upper compartment, or in both compartments. The results are presented as cells per 10 HPF and as the mean ± SEM of five different experiments.

Abbreviation: ND, not determined.

* Significantly different from baseline values at $P < .05$, Student's $t$ test.

Table 2. Chemotaxis of Unprimed and IL-5-Primed Normal Human Eosinophils Toward IL-8, RANTES, and MIP-1α at a Concentration of $10^{-8}$ mol/L

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>n</th>
<th>Unprimed</th>
<th>IL-5-Primed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>5</td>
<td>22.4 ± 4.3</td>
<td>119.7 ± 9.7*</td>
</tr>
<tr>
<td>RANTES</td>
<td>6</td>
<td>119.7 ± 9.7*</td>
<td>176.7 ± 8.7*</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>6</td>
<td>215.7 ± 7.4</td>
<td>74.0 ± 5.1</td>
</tr>
</tbody>
</table>

Results are presented as cells per 10 HPF and as the mean ± SEM of $n$ different experiments. Background migration toward incubation buffer in these experiments was 11.2 ± 1.8 for unprimed cells and 70.5 ± 1.9 for IL-5 primed cells ($n = 6$).

* Significantly different from baseline values at $P < .05$, Student's $t$ test.
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in both unprimed and IL-5–primed eosinophils, using the 15-second time point. Since only IL-8 and RANTES elicited a chemotactic response in IL-5–primed eosinophils, we studied whether these chemokines induced an increase in relative F-actin content. In Fig 6A, it is shown that RANTES induced a significant increase in relative F-actin content both in unprimed (10^{-9} to 10^{-7} mol/L, 99% confidence interval) and primed eosinophils (10^{-9} to 10^{-7} mol/L, 99% confidence interval). Figure 6B shows the dose-response curve for IL-8, demonstrating that IL-8 significantly induced actin polymerization at concentrations of 10^{-8} mol/L and 10^{-7} mol/L, (99% confidence interval) only after priming with IL-5. Table 3 shows the relative increase in F-actin content induced by the studied chemokines at a concentration of 10^{-8} mol/L. MCP-1, PF-4, and NAP-2 induced no significant changes in relative F-actin content, both in primed and unprimed eosinophils. Remarkably, MIP-1α (10^{-8} mol/L), which is not a chemotaxin for eosinophils, significantly induced actin polymerization in IL-5–primed cells (95% confidence interval), comparable to an optimal concentration of RANTES.

DISCUSSION

The proinflammatory cytokines of the chemokine family have a molecular weight of 8 to 10 kD and are structurally related by a conserved four-cysteine motif. The chemokine family is divided in a C-C branch and a C-x-C branch, according to the position of the first two cysteines in the conserved motif. For both branches, several receptors have been published. For instance, the receptor for the C-x-C chemokine IL-8 appears to belong to the family of G-protein coupled receptors, which contain seven transmembrane domains. Recently, a seven-transmembrane receptor called C-C CKR-1, with affinity for the C-C chemokines (RANTES, MCP-1, and MIP-1α/β), has been cloned and functionally characterized.

In this report, we studied the chemokines IL-8, NAP-2, and PF-4, belonging to the C-x-C branch, and RANTES, MCP-1, and MIP-1α, members of the C-C branch. As we previously reported, IL-8 was a chemotaxin for normal eosinophils after priming with GM-CSF, IL-3, or IL-5. However, we could not detect any chemotactic activity of NAP-2 and PF-4. We could not reproduce our earlier data describing migratory responses of eosinophils induced by PF-4, possibly due to contamination of this platelet-derived product with adenosine triphosphate (ATP) in an earlier batch. ATP has been shown to be the predominant chemotaxant for eosinophils in supernatants of thrombin-stimulated platelets. From the C-C chemokines, RANTES induced a significant migratory response in unprimed eosinophils only at a high concentration (10^{-6} mol/L, Table 2). However, it is unlikely that this concentration is present in vivo. Interestingly, after priming with IL-5, RANTES proved to be a chemotaxin at lower concentrations (10^{-9} to 10^{-7} mol/L). The same priming-induced leftward shift is induced when PAF is used as a chemotaxant. Hence, when eosinophils are primed in vivo in allergic subjects, the cells might become sensitive for lower concentrations of RANTES, and therefore this chemokine might be involved in the specific eosinophilic influx in allergic lesions.

Our observations seem to be in contrast with studies from several groups, who reported eosinophil migratory responses toward RANTES at concentrations of 10^{-8} and 10^{-7} mol/L, and to a lesser extent toward MIP-1α (10^{-6} mol/L), without the apparent need for priming. This difference is unclear, but several factors might play a role. We used a Boyden chamber assay with a cellulose nitrate filter with a thickness of 150 μm, while other studies used 10-μm thick polycarbonate filters. The latter filters might more easily detect migratory responses toward weak chemoattractants, but include the higher risk of bias by chemokinesis. In addition, other studies used eosinophils from the blood of normal donors and subjects with mild eosinophilia (5% to 10% eosinophils) or from normal donors and allergic subjects (4% to 8% eosinophils). The obtained results were combined and presented as one group. In vivo–primed cells derived from allergic donors or donors with eosinophilia might have influenced the results in these studies. Indeed, eosinophil–priming cytokines were demonstrated in the circulation of patients with allergic asthma and IL-5 was found in the circulation of patients with idiopathic hypereosinophilia. Rot et al have shown before that RANTES, MIP-1α, and, to a lesser extent, IL-8 are able to increase [Ca^{2+}] in human eosinophils, and that cross-desensitization was observed.
when RANTES (100 nmol/L) was followed by MIP-1α (50 nmol/L), but not the other way around. In this study, we could confirm these data, with the exception that IL-8 did not induce an appreciable increase in [Ca²⁺], even when 50 nmol/L was given to the cells (not shown). The heterologous desensitization (Fig 4) is an indication that a chemokine receptor on eosinophils, involved in the increases in [Ca²⁺], can bind both RANTES and MIP-1α. Indeed, the C-C CKR-1 chemokine receptor expressed in human kidney 293 cells, as well as the C-C chemokine receptor on the monocytic cell line THP-1, acts as a promiscuous receptor able to bind more than one C-C chemokine. Others have speculated on a two-receptor model on human eosinophils with one shared receptor for RANTES and MIP-1α and a RANTES-specific receptor, which might also be in line with the data. The fact that only RANTES is a chemoattractant for eosinophils might be explained by the existence of a specific RANTES receptor signal for migration, whose function is upregulated by priming with IL-5. Interestingly, priming with IL-5 did not result in enhancement of the [Ca²⁺] responses induced by the different chemokines. From our data, we can also conclude that chemokines that induce [Ca²⁺], mobilization do not necessarily induce a migratory response, since MIP-1α and NAP-2 were not able to induce eosinophil migration. Furthermore, IL-8 did not induce an appreciable increase in [Ca²⁺], but induced migration after priming with IL-5, indicating that [Ca²⁺], mobilization per se is not essential for eosinophil migration. This is in line with observations in neutrophils, where an elevation in [Ca²⁺], is not essential to induce migratory responses, since migratory responses toward formylpeptides were still present after Ca²⁺ depletion.

Since [Ca²⁺], responses do not directly correlate with eosinophil migration, we studied actin polymerization, which is another intracellular locomotory-associated event. We found that actin polymerizes rapidly in normal human eosinophils (within 10 to 15 seconds) after exposure to FMLP and PAF, both at 10⁻⁸ mol/L. Actin polymerization induced by these chemotaxins was significantly enhanced after priming with IL-5 (Fig 5), which nicely correlates with the sensitivity for priming of PAF-induced eosinophil migration. Therefore,
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Table 3. Actin Polymerization Induced by MIP-1α, MCP-1, PF-4, NAP-2, RANTES, and IL-8 at a Concentration of 10^-8 mol/L After 15 Seconds

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>n</th>
<th>Unprimed</th>
<th>IL-5-Primed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>9</td>
<td>1.07 (0.99-1.15)</td>
<td>1.24 (1.08-1.40)*</td>
</tr>
<tr>
<td>PF-4</td>
<td>4</td>
<td>1.10 (0.89-1.31)</td>
<td>1.12 (0.99-1.23)</td>
</tr>
<tr>
<td>NAP-2</td>
<td>4</td>
<td>1.15 (0.77-1.53)</td>
<td>1.18 (0.86-1.50)</td>
</tr>
<tr>
<td>RANTES</td>
<td>9</td>
<td>1.50 (1.39-1.62)*</td>
<td>1.76 (1.55-1.95)*</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>4</td>
<td>1.32 (0.92-1.72)</td>
<td>1.54 (1.11-1.97)*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4</td>
<td>1.10 (0.72-1.48)</td>
<td>1.08 (0.96-1.20)</td>
</tr>
</tbody>
</table>

Results are expressed as relative F-actin content, mean (99% confidence interval, except for PF-4 and MIP-1α, for which a 95% confidence interval was used) of n different experiments.

* Significantly different from baseline values.

we studied chemokine-induced actin polymerization to possibly correlate migratory effects of the chemokines with this intracellular signal. From the studied C-C chemokines, RANTES significantly induced actin polymerization in unprimed and IL-5-primed eosinophils, whereas MIP-1α was only active in IL-5-primed cells. From the C-X-C chemokines, only IL-8 induced a significant increase in relative F-actin content provided the cells had been primed with IL-5. These experiments were performed using a 15-second activation period, since chemoattractant-induced actin polymerization was maximal at that time point. Our data indicate that, in eosinophils, the receptor-mediated induction of both actin polymerization and an increase in [Ca^2+], simultaneously are not sufficient signals to elicit a migratory response, since we could not detect migratory activity toward MIP-1α. There seems to be an other yet unidentified signal involved in eosinophil migration, which is not activated by MIP-1α. On the other hand, it can be concluded that when chemokines elicit a chemotactic response in normal human eosinophils, it is accompanied by actin polymerization, since both RANTES and IL-8 do so.

This study provides further evidence that Th2-cell-derived cytokines like IL-5 and locally secreted chemokines like RANTES and IL-8 can contribute to the specific eosinophil influx into sites of allergic inflammation.

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RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5

RC Schweizer, BA Welmers, JA Raaijmakers, P Zanen, JW Lammers and L Koenderman