

## **Monocyte Chemotactic Protein 3 Is a Most Effective Basophil- and Eosinophil-activating Chemokine**

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### **Summary**

CC chemokines constitute a novel class of cytokines that attract and activate monocytes and lymphocytes, as well as basophil and eosinophil leukocytes, with distinct target cell profiles, and are believed to be involved in the regulation of different types of inflammation. The action of the recently identified monocyte chemotactic protein 3 (MCP-3) on human basophil and eosinophil function was studied and compared with that of other CC chemokines. In basophils, MCP-3, MCP-1, RANTES, and macrophage inflammatory protein (MIP)-1 $\alpha$  all induced cytosolic-free calcium concentration ( $[Ca^{2+}]_i$ ) changes and, with different efficacies, chemotaxis (RANTES = MCP-3  $\gg$  MCP-1  $>$  MIP-1 $\alpha$ ), histamine release (MCP-1 = MCP-3  $\gg$  RANTES  $>$  MIP-1 $\alpha$ ), and leukotriene C<sub>4</sub> formation, after IL-3 pretreatment (MCP-1 = MCP-3  $\gg$  RANTES  $>$  MIP-1 $\alpha$ ). Thus, MCP-3 was as effective as MCP-1 as an inducer of mediator release, and as effective as RANTES as a stimulus of basophil migration. In contrast to MCP-1, MCP-3 was also a stimulus for eosinophils, and induced  $[Ca^{2+}]_i$  changes and chemotaxis as effectively as RANTES, which is the most potent chemotactic cytokine for these cells. Desensitization of the transient changes in  $[Ca^{2+}]_i$  was used to assess receptor usage. In basophils, stimulation with MCP-3 prevented responsiveness to MCP-1 and RANTES, but not to MIP-1 $\alpha$ . No single CC chemokine (except for MCP-3 itself) affected the response to MCP-3, however, which was prevented only when the cells were prestimulated with both MCP-1 and RANTES. In eosinophils, by contrast, cross-desensitization between RANTES and MCP-3 was obtained. RANTES and to a lesser extent MCP-3 also desensitized eosinophils toward MIP-1 $\alpha$ . The desensitization data suggest the existence of three chemokine receptors: (a) a MCP-1 receptor expressed on basophils but not eosinophils that is activated by MCP-1 and MCP-3; (b) a RANTES receptor in basophils and eosinophils that is activated by RANTES and MCP-3; and (c) a MIP-1 $\alpha$  receptor that is activated by MIP-1 $\alpha$ , RANTES and, more weakly, by MCP-3. This study shows that MCP-3 combines the properties of RANTES, a powerful chemoattractant, and MCP-1, a highly effective stimulus of mediator release, and thus has a particularly broad range of activities toward both human basophil and eosinophil leukocytes.

A few years ago, chemotactic cytokines (now termed chemokines) were viewed as attractants for neutrophils (CXC chemokines) or mononuclear cells (CC chemokines). It was then found that IL-8 induces histamine and leukotriene (LT)<sup>1</sup>C<sub>4</sub> release from IL-3-primed human blood basophils (1, 2) via GTP-binding protein-coupled receptors (3). Later studies showed that some CC chemokines activate basophil as well as eosinophil leukocytes, suggesting that they may function

as mediators in allergic conditions and parasitic infestations. Several laboratories reported recently that monocyte chemotactic protein (MCP)-1 is a powerful stimulus of histamine release from human blood basophils (4–6). Priming of the cells with IL-3, IL-5, or GM-CSF enhanced histamine release and conditioned the cells to produce peptido-leukotrienes in response to MCP-1, which was considerably more potent and effective than IL-8 (4, 6). Significant but less pronounced release was observed in primed basophils upon stimulation with RANTES and macrophage inflammatory protein (MIP)-1 $\alpha$  (7–9). A direct comparison recently confirmed that MCP-1 is superior to RANTES as a stimulus of histamine and LTC<sub>4</sub>

<sup>1</sup> Abbreviations used in this paper:  $[Ca^{2+}]_i$ , cytosolic-free calcium concentration; LT, leukotriene; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein.

release, and showed that RANTES is considerably more potent than MCP-1 as a basophil chemoattractant (9). RANTES and MIP-1 $\alpha$  are also potent chemoattractants for eosinophil leukocytes, while MCP-1 is totally inactive on these cells (10).

A novel CC chemokine was recently identified in the supernatants of osteosarcoma cell cultures (11), and termed MCP-3 because of its marked sequence similarity with MCP-1. The cDNA coding for this chemokine was cloned and expressed (12, 13). We have now studied the effects of recombinant MCP-3 on human basophil and eosinophil leukocytes in comparison with MCP-1, RANTES, and MIP-1 $\alpha$ . Our results show that MCP-3 stimulates both types of leukocytes, inducing cytosolic-free calcium changes, chemotaxis, and release of histamine and LTC<sub>4</sub>. As highly effective chemoattractant and inducer of mediator release, MCP-3 combines the properties of MCP-1 and RANTES, and thus represents a most effective chemokine for basophil and eosinophil leukocytes.

## Materials and Methods

**Reagents.** Dextran and Ficoll-Hypaque were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); EDTA and fura-2/AM were from Fluka AG (Buchs, Switzerland); Hepes was from Calbiochem-Behring Corp. (La Jolla, CA); BSA (fatty acid free) was from Boehringer (Mannheim, FRG); ionomycin was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of highest purity available.

**Cytokines.** Recombinant human MCP-1, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were purchased from Prepro Tech Inc. (Rocky Hill, NJ) and MCP-3 was prepared as described by Minty et al. (12). Recombinant human IL-3 and IL-8 were kindly provided by Sandoz Ltd. (Basel, Switzerland and Vienna, Austria). All proteins were dissolved in 20 mM Hepes buffer, pH 7.4, containing 1 mg/ml BSA at 10<sup>-5</sup> M, and stored at -70°C.

**Cells.** Basophil and eosinophil leukocytes were prepared from freshly drawn venous blood of unselected healthy volunteers as described previously (6, 9). Basophils were purified by centrifugation on a discontinuous Percoll gradient followed by negative selection with magnetic beads coated with mAbs against CD3, CD4, CD8, CD14, CD16, and CD19 (9). The final preparation consisted of 80–95% basophils and 5–20% small lymphocytes, and the recovery was 30–60%. Eosinophils were purified to 99.5% by combining Percoll density gradient centrifugation and negative selection with anti-CD16 mAb-conjugated immunomagnetic beads (10).

**Histamine and LTC<sub>4</sub> Release.** Basophils (80–180 × 10<sup>3</sup>/ml) in 20 mM Hepes, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM glucose, and 0.025% BSA were warmed to 37°C, exposed to IL-3 (10 ng/ml) or vehicle for 10 min, and then challenged with a chemokine. After 20 min, the tubes were placed in ice, and histamine and LTC<sub>4</sub> were measured in the cell supernatant (14). Histamine release was expressed in percent of the total content of the sample (determined after cell lysis). LTC<sub>4</sub> generation was expressed as picograms LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> per nanogram total histamine (which corresponds to ~1,000 basophils).

**Chemotaxis.** Chemotactic chambers with 48-well (Neuro Probe, Cabin John, MD) and polyvinyl-pyrrolidone-free polycarbonate filters with 5- $\mu$ m pores (Nucleopore, Pleasanton, CA) were used and the assays performed as described previously (9, 10). After incubation at 37°C in 5% CO<sub>2</sub> for 60 min the filter was removed,

washed, fixed, and stained, and the migrated cells were counted in five randomly selected fields of 0.03 mm<sup>2</sup>.

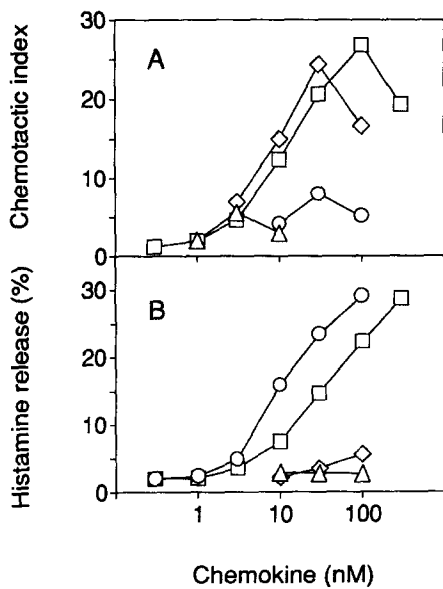
**Cytosolic-free Calcium ([Ca<sup>2+</sup>]<sub>i</sub>) Changes.** Purified eosinophils or basophils were loaded with fura-2/AM (0.3 nmol/10<sup>6</sup> cells) in 20 mM Hepes, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM glucose, and 0.025% BSA for 30 min at 37°C, and [Ca<sup>2+</sup>]<sub>i</sub> changes were determined after chemokine stimulation (9). In all experiments, stimulation with CC chemokines was followed by IL-8 and C5a as controls that showed preserved responsiveness of the cells to these two agonists. After each measurement maximum and minimum fluorescence was calibrated by addition of 5  $\mu$ M ionomycin followed by 1 mM MnCl<sub>2</sub>.

## Results

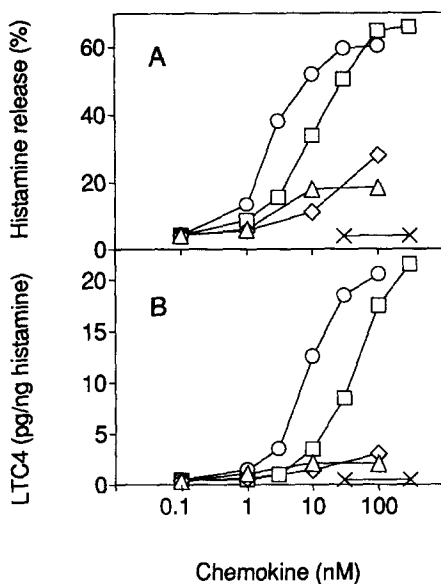
**Activation of Basophils and Eosinophils.** All four chemokines tested induced the migration of human blood basophils. As shown in Fig. 1 A, MCP-3 and RANTES were highly effective and almost equally potent. Significant, but considerably weaker migration was observed with MCP-1 and MIP-1 $\alpha$ . Of the four active chemokines, MIP-1 $\alpha$  had the lowest efficacy, while its homologue MIP-1 $\beta$  was inactive as shown previously (9). MCP-1 and MCP-3 also induced a marked, concentration-dependent release of histamine by cells that were not primed with IL-3 or another myeloid growth factor. In cells from unselected donors, as used in this study, RANTES showed only borderline effects and MIP-1 $\alpha$  was inactive (Fig. 1 B). Exocytosis was very rapid and virtually complete within 1 min after stimulation with MCP-3 or the other chemokines. No CC chemokine induced the generation of LTC<sub>4</sub> in unprimed cells.

Priming with IL-3 expectedly enhanced the sensitivity of the cells as shown by a shift to the left of the concentration dependence curve, the higher amounts of histamine released, and the production of LTC<sub>4</sub> (Fig. 2). MCP-1 and MCP-3 were about equally active and considerably more effective than RANTES and MIP-1 $\alpha$ , which induced only low levels of release of either product. Since IL-3-primed basophils are particularly sensitive to stimulation, MIP-1 $\beta$  was also tested, but no activity was found (Fig. 2).

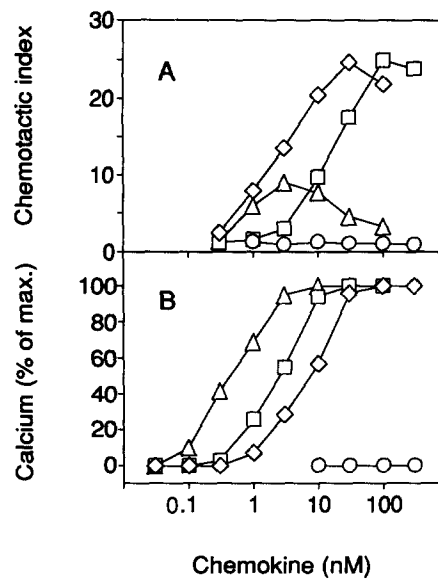
MCP-3, RANTES, and MIP-1 $\alpha$  induced chemotaxis and a rise in cytosolic-free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in human eosinophils, while MCP-1 was completely inactive (Fig. 3). Although RANTES and MIP-1 $\alpha$  were chemotactic at lower concentrations, MCP-3 showed high efficacy and induced the migration of similar numbers of cells as RANTES. The maximal chemotactic index obtained for eosinophils and basophils after stimulation with either MCP-3 and RANTES was well above 20 in all experiments performed. As previously shown for RANTES (10), the migration of basophils and eosinophils towards MCP-3 was due to a chemotactic rather than chemokinetic effect, since there was little migration when the chemokine was placed on both sides of the filter (data not shown). The activities of RANTES and MIP-1 $\alpha$  are in agreement with former observations (10), but the high effectiveness of MCP-3 was surprising since this chemokine shares ~70% sequence identity with MCP-1, which does not stimulate eosinophils.



**Figure 1.** Basophil activation by MCP-3 and related CC chemokines. (A) Chemotaxis in vitro. Each point represents the chemotactic index for the mean migration value from two experiments performed in triplicate with basophils from different unselected donors. MCP-1 and MIP-1 $\alpha$  were tested at three concentrations only, within the optimal range established previously (9). (B) Histamine release from unprimed basophils in 20 min. Each point represents the mean value from three experiments performed in duplicate with basophils from different unselected donors. No LTC<sub>4</sub> release was observed under these conditions. MCP-3 (□); MCP-1 (○); RANTES (◇); and MIP-1 $\alpha$  (Δ).



**Figure 2.** Histamine and LTC<sub>4</sub> release by IL-3-primed human basophils after stimulation with MCP-3 and related CC chemokines. The cells were pretreated for 10 min with 10 ng/ml IL-3, stimulated with chemokines, and the release of histamine (A) and LTC<sub>4</sub> (B) was determined after 20 min. Mean values from three experiments performed with basophils from different unselected donors. MCP-3 (□); MCP-1 (○); RANTES (◇); MIP-1 $\alpha$  (Δ); and MIP-1 $\beta$  (×).

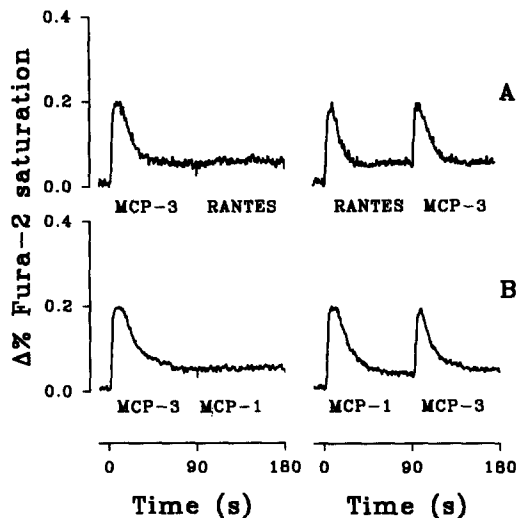


**Figure 3.** Eosinophil activation by MCP-3 and related CC chemokines. (A) Chemotaxis in vitro. Each point represents the chemotactic index for the mean migration value from two experiments performed in triplicate with eosinophils from different unselected donors. Similar results were obtained in three additional experiments. (B) [Ca<sup>2+</sup>]<sub>i</sub> changes (assessed as fura-2 saturation) in percent of maximum rise obtained after stimulation with RANTES. Mean values from three experiments performed with eosinophils from different unselected donors. MCP-3 (□); MCP-1 (○); RANTES (◇); and MIP-1 $\alpha$  (Δ).

**Receptors.** Earlier studies had suggested that basophil and eosinophil leukocytes bear different types of CC chemokine receptors (9, 10). Desensitization experiments were, therefore, performed to gain information on the types of receptors involved in the activation by MCP-3. To compare basophil and eosinophil responses, [Ca<sup>2+</sup>]<sub>i</sub> changes were used to assess receptor activation.

As shown in Fig. 4, stimulation with MCP-3 abrogated the response of basophils to a subsequent stimulation with either RANTES or MCP-1. The [Ca<sup>2+</sup>]<sub>i</sub> rise induced by MCP-3, by contrast, was not affected by prior stimulation with MCP-1, RANTES, or MIP-1 $\alpha$  (data for MIP-1 $\alpha$  not shown). The effect of sequential additions of several chemokines was then studied. As shown in Fig. 5, the [Ca<sup>2+</sup>]<sub>i</sub> rise induced by MCP-3 was prevented only when the cells were first stimulated with the combination of MCP-1 and RANTES (Fig. 5 A). Prestimulation with MCP-1 and MIP-1 $\alpha$  or with MIP-1 $\alpha$  and RANTES did not abolish the MCP-3 response. The effects as described were independent of the order of addition of the two chemokines before MCP-3, and in all cases, basophils remained responsive to IL-8, indicating that [Ca<sup>2+</sup>]<sub>i</sub> changes could still be induced by stimulation via CXC chemokine receptors.

Together, the results of these experiments indicate that MCP-3 acts on basophils via two receptors, one with selectivity for MCP-1, and the other with selectivity for RANTES. The cells remained responsive to MCP-3 when only one of the receptors was desensitized (prestimulation with MCP-1 or RANTES), but became unresponsive when desensitization

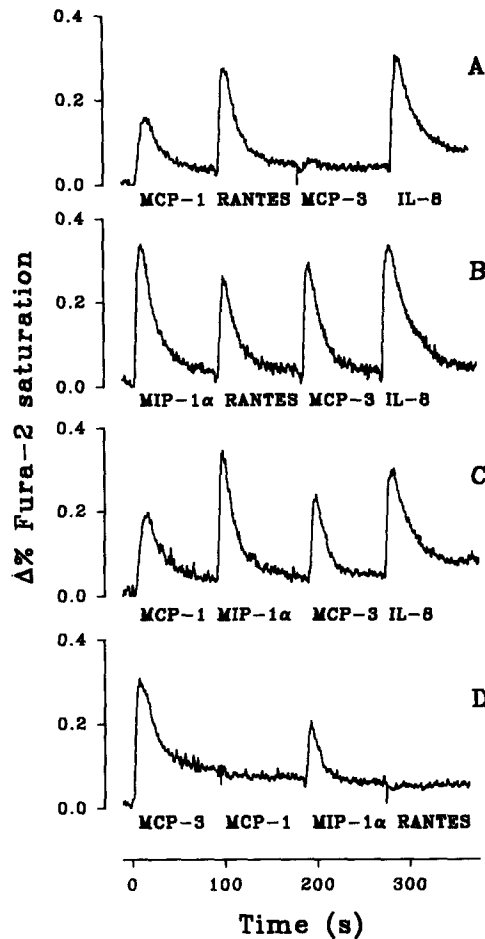


**Figure 4.** Cross-desensitization of basophils stimulated with MCP-3, MCP-1, and RANTES. Fura-2-loaded cells ( $10^6/\text{ml}$ ) were sequentially stimulated with 50 nM chemokine at 90-s intervals, and the  $[\text{Ca}^{2+}]_i$ -dependent fluorescence changes were recorded. All stimulus sequences were tested at least three times with cells from different unselected donors.

affected both (prestimulation with MCP-1 and RANTES). The finding that stimulation with MCP-3 prevented basophil responses to either MCP-1 or RANTES (Fig. 4), but not to MIP-1 $\alpha$  (Fig. 5 D) suggests the presence of a third receptor with selectivity for MIP-1 $\alpha$ . The desensitization patterns indicate that the situation is somewhat different for eosinophils. It was known from a former study that these cells do not respond to MCP-1 (10), and in fact, we observed no  $[\text{Ca}^{2+}]_i$  changes and a subsequent challenge with MCP-3 was not affected when eosinophils were exposed to MCP-1 (data not shown). If eosinophils lack a receptor for MCP-1, the action of MCP-3 could be mediated by the RANTES receptor, in which case cross-desensitization between MCP-3 and RANTES would be expected. Fig. 6 A shows that this is indeed the case. In agreement with a previous study (10), desensitization was also observed when eosinophils were stimulated with RANTES followed by MIP-1 $\alpha$ , but not vice versa (Fig. 6 C), indicating that RANTES also acts on the MIP-1 $\alpha$  receptor. MCP-3, however, affected only slightly the response of eosinophils to MIP-1 $\alpha$  (Fig. 6 B), suggesting that it interacts with the MIP-1 $\alpha$  receptor less effectively than RANTES. This may explain why RANTES prevented the response to MCP-3, while MCP-3 did not completely abolish the response to RANTES (Fig. 6 A). The response to RANTES was abrogated by combined prestimulation with MCP-3 and MIP-1 $\alpha$ , further indicating that RANTES acts on eosinophils via two distinct receptors (data not shown).

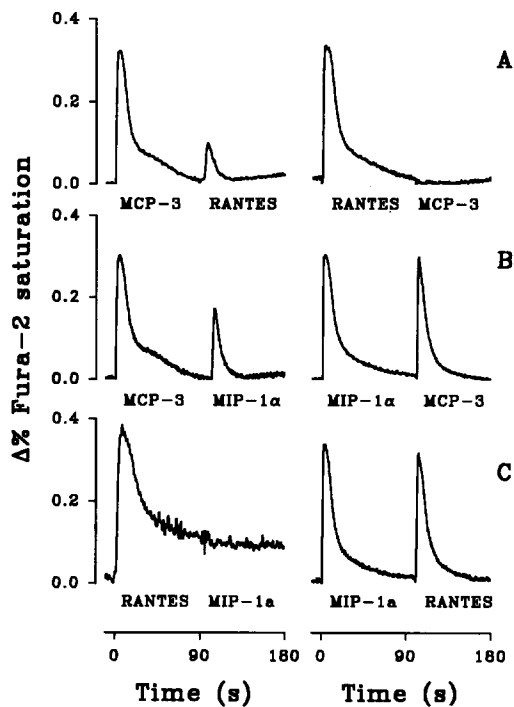
## Discussion

Recent studies have indicated that CC chemokines are powerful stimuli of basophil and eosinophil leukocytes. Three chemokines act on basophils: MCP-1 induces preferentially mediator release (4-6), while RANTES and MIP-1 $\alpha$  are more



**Figure 5.** Desensitization of basophils by sequential stimulation with CC chemokines. Fura-2-loaded cells ( $10^6/\text{ml}$ ) were sequentially stimulated with two CC chemokines and then with MCP-3. IL-8 was used as the last stimulus to show that the cells could still respond by mobilizing  $\text{Ca}^{2+}$ . All chemokines were used at 50 nM.  $[\text{Ca}^{2+}]_i$ -dependent fluorescence changes are shown. All stimulus sequences were tested at least three times with cells from different unselected donors, and identical desensitization patterns were obtained. The only variability observed was a slight donor-to-donor difference in the maximal  $[\text{Ca}^{2+}]_i$  levels induced by different chemokines, in particular by MCP-1 (compare Figs. 4 and 5).

effective as chemoattractants (7-9). RANTES and MIP-1 $\alpha$  also activate eosinophils (10, 15). It was, therefore, of interest to study the effects of MCP-3, a novel CC chemokine structurally related to MCP-1 (11-13). We have found that MCP-3 has the broadest spectrum of activity of all chemokines studied; it was as effective as MCP-1 as an inducer of mediator release in basophils, and as effective as RANTES as a stimulus of basophil and eosinophil migration. The fact that MCP-3 induced histamine release with similar efficacy to MCP-1 was expected because these chemokines share >70% sequence identity. Its potent chemotactic activity, and in particular its action on eosinophils, were surprising, however, since the sequences of MCP-3 and RANTES are only distantly related (25% sequence identity). It thus appears that sequence homology is not necessarily predictive for the capacity of different CC chemokines to activate one or the other effector func-



**Figure 6.** Cross-desensitization of eosinophils stimulated with MCP-3, RANTES, and MIP-1 $\alpha$ . Fura-2-loaded cells ( $10^6$ /ml) were stimulated with 50 nM chemokine, and the  $[Ca^{2+}]_i$ -dependent fluorescence changes were recorded. All stimulus sequences were tested four times with cells from different unselected donors.

tions in basophils and eosinophils, and that activity is more likely to depend on discrete sequence motifs.

Desensitization analysis with real-time recording of a rapid response like the transient change in  $[Ca^{2+}]_i$  is a sensitive way to assess receptor usage by related agonists, and the method of choice when only low numbers of cells are available, as in the present study. In such experiments evidence was obtained for the existence of distinct receptors for MCP-1 and RANTES on basophils (9, 10), and the present results suggest that MCP-3 not only shares the biological activities of two related chemokines, but also interacts with their receptors. Stimulation with MCP-3 abrogated responsiveness to MCP-1 and RANTES. To prevent the response to MCP-3, however, prestimulation with both MCP-1 and RANTES was required. In eosinophils (which do not respond to MCP-1 and do not appear to express MCP-1 receptors) by contrast, RANTES alone was sufficient to fully desensitize the cells towards MCP-3.

In addition to the two receptors that are shown here to mediate the effects of MCP-3, our previous studies (9, 10) had suggested the existence of a third CC chemokine receptor in basophils and eosinophils with a high affinity for MIP-1 $\alpha$  and a somewhat lower affinity for RANTES. MCP-3 was less efficient than RANTES in desensitizing the cells toward MIP-1 $\alpha$ , suggesting that RANTES is a better ligand. This MIP-1 $\alpha$  receptor may correspond to a recently cloned CC chemokine receptor (16, 17) that binds MIP-1 $\alpha$  with high affinity and also interacts with RANTES. A MIP-1 $\alpha$  receptor is also present in human neutrophils, where MIP-1 $\alpha$  and to

a lesser extent RANTES were shown to elicit  $[Ca^{2+}]_i$  changes, but no chemotaxis or exocytosis (17, 18). We have found that MCP-3 induces similar  $[Ca^{2+}]_i$  changes, but no functional responses in neutrophils, and that these changes are prevented by prestimulation with MIP-1 $\alpha$  (data not shown). On the other hand, in the present study, the responses of basophils to MCP-1 and MCP-3, and of eosinophils to RANTES and MCP-3 were not affected by prestimulation with MIP-1 $\alpha$ , suggesting that the recently cloned receptor for MIP-1 $\alpha$  mediates other functions.

Our results suggest the existence of three chemokine receptors: (a) a MCP-1 receptor expressed on basophils but not eosinophils that is activated by MCP-1 and MCP-3, and mediates predominantly mediator release; (b) a RANTES receptor in basophils and eosinophils that is activated by RANTES and MCP-3, and mediates mainly chemotaxis; and (c) a MIP-1 $\alpha$  receptor in basophils, eosinophils, and neutrophils that is activated by MIP-1 $\alpha$ , RANTES, and, more weakly, by MCP-3. The function of the MIP-1 $\alpha$  receptor is still unclear. These conclusions are so far largely based on functional assays and desensitization studies, and other interpretations are also possible. Heterologous desensitization of calcium transients induced by C5a and FMLP has been reported, particularly when the time interval between the two agonists is 5 min or more, and when the concentration of the second stimulus is suboptimal (19). We, therefore, cannot exclude the presence of additional receptors (e.g., MCP-3-specific receptors) that cross-deactivate other CC chemokine receptors, although we observed virtually no cross-desensitization of calcium transients in different granulocyte types sequentially stimulated with a large number of different agonists (CC chemokines, IL-8, C5a, C3a, FMLP), provided that they are used at high (50–100 nM) concentrations within short (60–90 s) time intervals (3, 6, 9, 10; and our unpublished observations). Lack of desensitization between two chemoattractants, however, strongly indicates activation through distinct receptors since we always observe full desensitization upon sequential exposure to the same agonist, although one cannot definitely exclude the possibility that a receptor occupied by one agonist can still be activated with another distinct ligand. Nevertheless, deactivation studies as performed here, particularly when combined with functional studies, appeared to be surprisingly predictive for the ligand selectivities of subsequently cloned chemoattractant receptors. For example, the recently cloned MIP-1 $\alpha$  receptor is also activated by RANTES (16, 17) as suggested in our previous studies (9, 10), despite the fact that MIP-1 $\alpha$  binding is displaced by RANTES no more efficiently than by other CC chemokines such as MCP-1 (16). Thus, deactivation of calcium transients can even give information that is not obtainable with studies of equilibrium binding at 4°C. We therefore believe that the model proposed above provides a reasonable and likely explanation of our observations, and gives a minimal estimate of different CC chemokine receptors present on basophils and eosinophils. However, additional information on ligand binding and cell activation is certainly needed using cells that express single CC chemokine receptors isolated by cloning.

Owing to their effects on mononuclear cells, basophils,

and eosinophils, CC chemokines must be regarded as major potential mediators of effector cell recruitment and activation in different types of chronic inflammation. Their actual involvement in the pathogenesis of such conditions will depend, in addition, on their expression in the affected tissues. In this regard, it is interesting that a novel murine chemokine with homology to MCP proteins, which was termed MARC, is expressed preferentially by murine mast cells and mast cell lines (20). The 3' noncoding region of the MARC and human MCP-3 cDNAs are highly homologous, suggesting that

MARC may be the murine analogue of human MCP-3 (12). MCP-3 may, therefore, play a major role in the pathogenesis of hypersensitivity diseases such as asthma and parasitic infestations.

MCP-1 and RANTES are CC chemokines with clearly distinct profiles of activity. The present results underline the potential importance of MCP-3 as an activator and an attractant of inflammatory cells since it combines, at least on basophils and eosinophils, the biological activities of MCP-1 and RANTES.

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