

Characterization and Species Distribution of High Affinity GTP-coupled Receptors for Human Rantes and Monocyte Chemoattractant Protein 1

By Gail Van Riper,* Salvatore Siciliano,† Paul A. Fischer,‡
Roger Meurer,§ Martin S. Springer,‡ and Hugh Rosen*

From the *Departments of Biochemical and Molecular Pathology, †Immunology Research, and
‡Cellular and Molecular Pharmacology, Merck Research Laboratories, Rahway, New Jersey
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Summary

Equilibrium binding studies with recombinant human chemoattractant cytokines Rantes and monocyte chemoattractant protein 1 (MCP-1) on monocytic THP-1 cells have allowed the functional identification of two distinct receptors for C-C chemokines. One is a novel oligospecific receptor with high affinity for Rantes (50% maximal inhibitory concentration [IC₅₀], 0.68 nM) and low affinity (IC₅₀, 35 nM) for MCP-1, while the other is the previously described specific receptor for MCP-1 (IC₅₀, 0.5 nM). Receptor affinity for Rantes is enhanced on preparation of isolated membranes with a 12-fold decrease in receptor K_d. The basis of this enhancement is not understood. The Rantes receptor appears to be G protein linked, as binding activity is abolished by guanosine 5'-O-(3-thiotriphosphate) (IC₅₀, 7.3 nM). In contrast to the consequences of MCP-1 binding, we were unable to demonstrate ligand-dependent calcium fluxes on binding of Rantes to human monocytes or THP-1 cells. The binding of Rantes and MCP-1 to mononuclear cells from dog, rabbit, and rat were tested. While high affinity binding could be demonstrated in dog and rabbit, differences in ligand-induced Ca²⁺ fluxes could be shown between species. This suggests that receptor-ligand interactions and receptor coupling is best examined with autologous receptors and cytokine.

Understanding of the molecular basis of the focal recruitment of monocytes and lymphocytes to sites of chronic inflammation remains incomplete. Identification of leukocyte-endothelial adhesion and homing molecules has provided some molecular insights into the inflammatory recruitment of monocytes (1), the recirculation of naive lymphocytes through high endothelium, and the induced migration of activated and memory T cells through unspecialized postcapillary venule (2). The recent convergence of studies on chemotactic cytokines, gene expression in activated leukocytes, and leukocyte adhesion molecules provide tools to dissect these interrelationships governing inflammatory leukocyte recruitment.

Rantes and monocyte chemoattractant protein 1 (MCP-1) are members of the intercrine (3) or chemokine (4) family of proinflammatory basic chemoattractant polypeptides. These cytokines, defined initially as genes expressed in activated leukocytes or as small platelet-derived growth factor (PDGF)-inducible genes, are structural members of the C-C branch of the chemokine family (5), based on the adjacent position of the first two of a highly conserved four-cysteine motif (5).

Both MCP-1 and Rantes are cell specific in their activation or chemoattractant activities. MCP-1 activity is restricted to

monocytes and basophils (6–8), while Rantes has been reported to be chemotactic for both monocytes, eosinophils (9) and the UCHL1-positive (low molecular weight CD45 isoform) memory subset of T cells (10). MCP-1 is expressed at sites of monocyte infiltration into tissues, such as early atherosclerotic lesions, or after vascular injury (11–13), and has been postulated to contribute to monocyte migration to such sites.

The biological and pathological contributions of these chemokine family members to inflammation and tissue repair remain to be elucidated experimentally, although a variety of proinflammatory effects have apparently been demonstrated by the injection of these cytokines *in vivo* (14). These include inflammatory recruitment of either neutrophils or mononuclear cells, depending upon the specific cytokine. It has been suggested, without causal evidence, that these molecules play an important role in inflammatory recruitment leading to cell activation and directional migration of specific leukocyte subsets, and contribute to activation of integrin-mediated adhesive events necessary for transendothelial migration (2).

Analysis of chemokine receptors is helpful in correlating levels of cytokine expression with potential effects and is also

an essential prerequisite to allow the adequate interpretation of the *in vivo* contribution of cytokines to inflammatory and immune pathology. To help clarify the relationship between chemokine presence and cell migration, we have characterized the human, rabbit, and dog receptors for Rantes and MCP-1, and demonstrate complexities of receptor-ligand relationships and receptor coupling among this family of molecules.

Materials and Methods

Cells and Membrane Preparation. THP-1 cells were cultured in IMDM with 10% FCS (Gibco Laboratories, Grand Island, NY). Plasma membranes were prepared by nitrogen bomb cavitation and differential centrifugation as described (15). Membrane preparations were resuspended at 3.3–3.5 mg/ml in 10 mM Hepes, pH 7.50, with PMSF, aprotinin, chymostatin, and leupeptin (all at 10 μ g/ml).

MCP-1 and Rantes. Recombinant ligands were purchased from Peptotech (Princeton, NJ) and iodinated with chloramine-T according to Rollins and Springer (16). Specific activities were 45 and 46 μ Ci/ μ g for MCP-1 and Rantes, respectively.

Binding Assays. Binding of $1\text{--}2 \times 10^4$ cpm of 125 I ligands in the presence of varying concentrations of unlabeled ligand to cells or membranes at room temperature was assayed in 50 mM Hepes, 1 mM CaCl_2 , 5 mM MgCl_2 , 0.5% BSA, pH 7.2. Activity retained on polyethylenimine-treated Whatman GFC filters after washing in binding buffer with 0.5 M NaCl was counted in a gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Binding constants were calculated and Scatchard analysis was performed on competition binding assays using the LIGAND program.

Inhibition of Binding by Guanosine 5'-O-(3-Thiotriphosphate) (GTP γ S). The binding of a constant concentration of 125 I ligand to THP-1 plasma membranes preincubated for 30 min at room temperature in varying concentrations of GTP γ S or ATP (Boehringer-Ingelheim) in binding buffer was assayed as above.

Ligand-induced Ca^{2+} Fluxes. Human or dog PBMC were prepared by density centrifugation on 1.077 g/ml Ficoll-Hypaque. Rabbit cells were prepared on 1.084 g/ml Percoll. These cells along with various monocytic cell lines (THP-1, U937, or Monomac6) were incubated with 1.25 μ g/ml Indo-1 in RPMI 1640, 10 mM Hepes, 5% FCS for 60 min at 37°C. Loaded cells were washed by centrifugation and warmed to 37°C before varying concentrations of ligand were added. Calcium analyses were performed on a FACS[®] analyzer (Becton Dickinson & Co., Mountain View, CA) with an excitation wavelength of 365 nm and dual emission wavelengths of 405 and 488 nm.

Results

Rantes Binding to THP-1 Cells and Membranes. Competition binding studies on intact THP-1 cells identified a single high affinity receptor with an IC_{50} of 0.69 nM (K_d , 0.39 nM) (Fig. 1 *a*) and $\sim 5,100$ binding sites/cell on Scatchard analysis. Preparation of isolated membranes from THP-1 cells resulted in the retention of a single high affinity binding site with an IC_{50} of 0.05 nM. Scatchard analysis revealed that the K_d of the receptor had decreased to 13 pM (Fig. 1 *b*). Neither IL-8 nor C5a competed for Rantes binding. The binding properties of the Rantes receptor are summarized in Table 1.

GTP γ S Sensitivity of Rantes Binding. Direct evidence of the GTP dependence and thus the G protein coupling of the Rantes receptor was provided by the GTP γ S sensitivity of

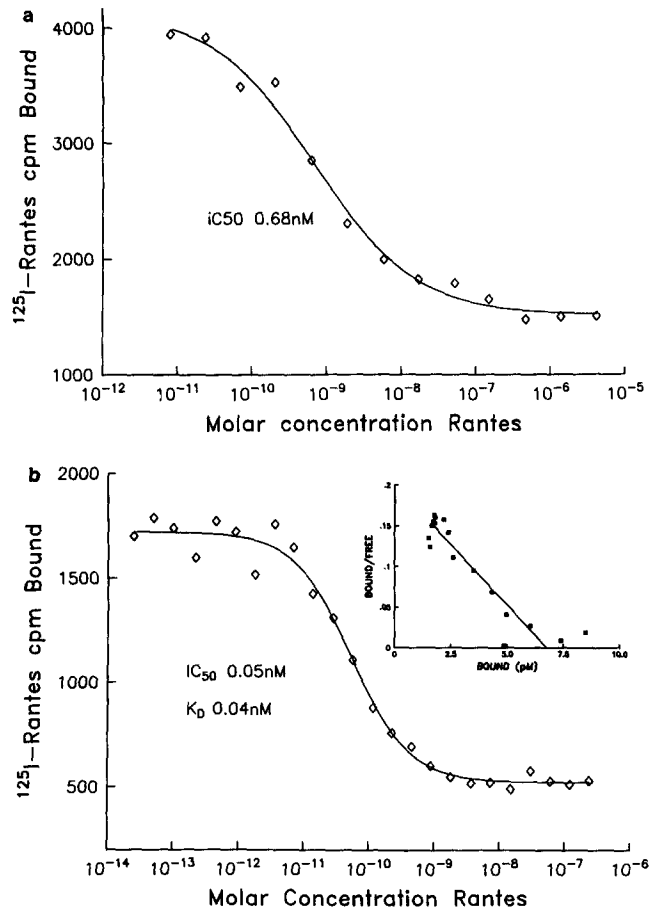


Figure 1. Equilibrium binding cold displacement curve of one of three essentially similar experiments of 125 I-Rantes binding to THP-1 cells (*a*) or isolated THP-1 membranes (*b*) showing the increase in receptor affinity upon cell disruption. The points represent the means of triplicate measurements with a superimposed four-parameter fit. The Scatchard plot for the membrane preparation (*inset*) shows a single high affinity receptor for 125 I-Rantes with a K_d of 13 pM compared with 0.19 nM for THP-1 cells.

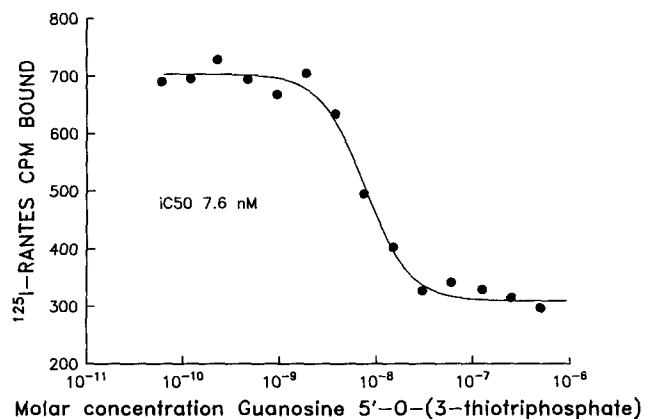


Figure 2. GTP γ S inhibits the binding of Rantes to its high affinity receptor with an IC_{50} of 7.6 nM. Membranes preincubated with varying concentrations of GTP γ S were then incubated in a constant amount of 125 I-Rantes. Points represent the means of triplicate measurements with a superimposed four-parameter fit. Essentially similar data were obtained in each of two separate experiments. Adenine nucleotides were inactive.

Table 1. Functional Characteristics Cells Surface Receptors for Rantes and MCP-1

Receptor	Ligand	K_d		B_{max} (sites/cell)	GTP γ S (IC ₅₀)
		Cells	Membranes		
		<i>nM</i>			
RANTES	RANTES	0.96 ± 0.29 (n = 4)	0.02 ± 0.015 (n = 3)	5,070 ± 108	7.3 nM
RANTES	MCP-1	105 ± 29	ND	3,010	ND
RANTES	IL-8 or C5a	No binding	No binding	ND	ND
MCP-1	MCP-1	0.51 ± 0.36 (n = 4)	0.33 ± 0.16 (n = 3)	3,923 ± 1,285	32 nM
MCP-1	RANTES	No binding	No binding	ND	ND
MCP1	IL-8 or C5a	No binding	No binding	ND	ND

Rantes binding to isolated THP-1 membranes. Fig. 2 shows that GTP γ S addition to membranes led to a concentration-dependent inhibition of Rantes binding with an IC₅₀ of 7.6 nM, an inhibition comparable to the GTP sensitivity of other high affinity G-linked receptors.

The High Affinity Rantes Receptor Has a Low Affinity for MCP-1 and Is Distinct from the High Affinity MCP-1 Receptor.

Crosscompetition experiments using ¹²⁵I-Rantes and unlabeled MCP-1 or Rantes on THP-1 cells showed that MCP-1 competed for the binding of iodinated Rantes with an IC₅₀ of 35 nM (Fig. 3 a). This IC₅₀ was between 35- and 50-fold greater than the IC₅₀ for unlabeled Rantes (Fig. 1 a). In contrast, competition experiments on THP-1 cells using ¹²⁵I-MCP-1 and unlabeled MCP-1 identified a high affinity MCP-1

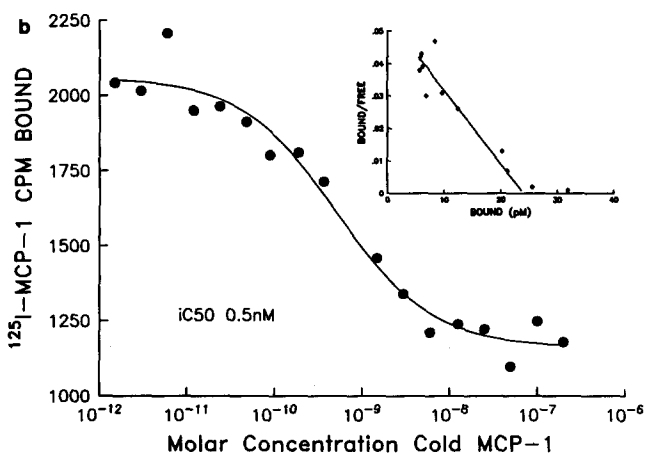
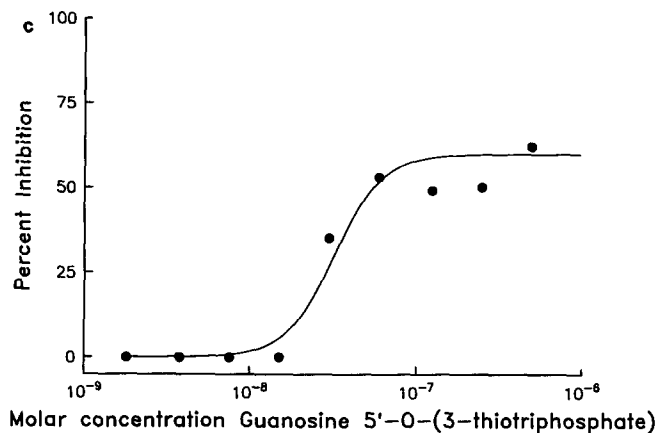
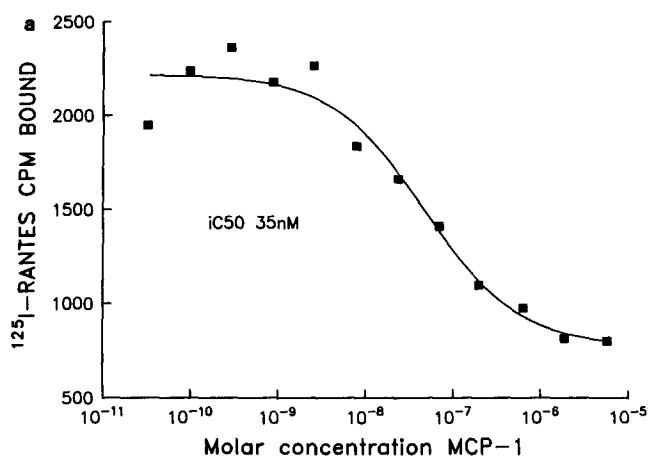


Figure 3. (a) Equilibrium binding cold displacement by MCP-1 of ¹²⁵I-Rantes binding to THP-1 cells demonstrating that MCP-1 binds with low affinity to the high affinity Rantes receptor. The points represent the means of triplicate measurements with a superimposed four-parameter fit. Essentially similar data were obtained in each of three separate experiments. (b) The binding of ¹²⁵I-MCP-1 to THP-1 cells is high affinity and distinct from the displacement of ¹²⁵I-Rantes by cold MCP-1. The points represent the means of triplicate measurements with a superimposed four-parameter fit. Essentially similar data were obtained in each of three separate experiments. An IC₅₀ of 0.5 nM for unlabeled MCP-1 competition for ¹²⁵I-MCP-1 binding was obtained compared with the IC₅₀ of 35 nM for unlabeled MCP-1 competition for ¹²⁵I-Rantes. Scatchard plot (*inset*) shows that THP-1 cells have a single high affinity receptor for ¹²⁵I-MCP-1 with a K_d of 0.44 nM. (c) GTP γ S inhibits the specific high affinity binding of MCP-1 to isolated THP-1 cell membranes. Essentially similar data were obtained in each of two separate experiments. Adenine nucleotides were inactive.

receptor with an IC_{50} of 0.5 nM (K_d of 0.44 nM) and 5,000 sites/cell (Fig. 3 b) when competed with MCP-1, while Rantes, IL-8, and C5a failed entirely to displace iodinated MCP-1 from its binding site. Furthermore, the inhibitory effect of GTP γ S on ^{125}I -MCP-1 binding to isolated membranes shows an IC_{50} of 32 nM, and is fourfold less sensitive to inhibition than the binding of ^{125}I -Rantes (Fig. 3 c). The Rantes receptor thus appears oligospecific, binding Rantes with high affinity and MCP-1 with low affinity, while the high affinity MCP-1 receptor is distinct and monospecific and appears not to share any binding activity for Rantes. The comparison of the binding properties of the two receptors are summarized in Table 1.

The Different Species Distribution of Human MCP-1 and Rantes Binding. Human MCP-1 differs significantly from rabbit, mouse, and rat MCP-1 in the absence of 25–48 COOH-terminal amino acids. Despite this, ^{125}I recombinant human MCP-1 bound to human, rabbit (K_d , 0.04 nM), dog, and pig mononuclear cells (Fig. 4). However, there was no demonstrable binding of human MCP-1 to rat cells, in spite of the reported *in vivo* activity of the natural human MCP-1 in rat (14). A specific binding signal on human, rat, dog, and rabbit cells was demonstrated with recombinant Rantes (Fig. 4).

High Affinity Rantes Binding Is Not Necessarily Coupled to a Ligand-induced Ca^{2+} Flux. The demonstration of either Rantes or MCP-1 binding activity does not always lead to the activation of a Ca^{2+} flux. Analysis of intracellular calcium transients upon binding of 1–100 nM MCP-1 led to a concentration-dependent transient Ca^{2+} flux in both THP-1 cells (Fig. 5 a) and human monocytes, whereas binding of Rantes failed to elicit a Ca^{2+} flux in either peripheral blood monocytes or THP-1 cells. Neither human Rantes nor human MCP-1 binding were able to induce a Ca^{2+} flux (Fig. 5 b) in rabbit cells, despite high affinity binding. In contrast, both Rantes and MCP-1 binding activated a transient Ca^{2+} flux in dog monocytes (Fig. 5 c), although the concentration of Rantes necessary to induce a significant flux in dog mono-

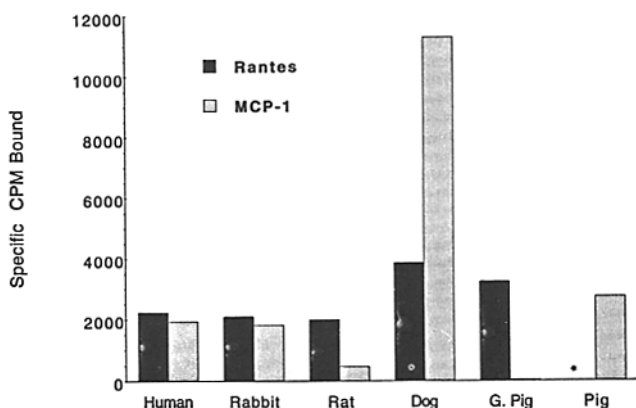


Figure 4. Summary of the binding data obtained using human MCP-1 and Rantes on mononuclear cells from various animal species. The bars represent the means of triplicate measurements after subtraction of the nonspecific binding (not competed by 100-fold excess of unlabeled cytokine). Essentially similar data were obtained in each of three separate experiments. *Binding of human Rantes to pig mononuclear cells was not determined.

cytes was 100 nM in comparison with 10 nM for MCP-1. The transient Ca^{2+} flux does not appear to be an essential component of the chemotactic response since Rantes induced human monocyte chemotaxis in chamber assays (not shown).

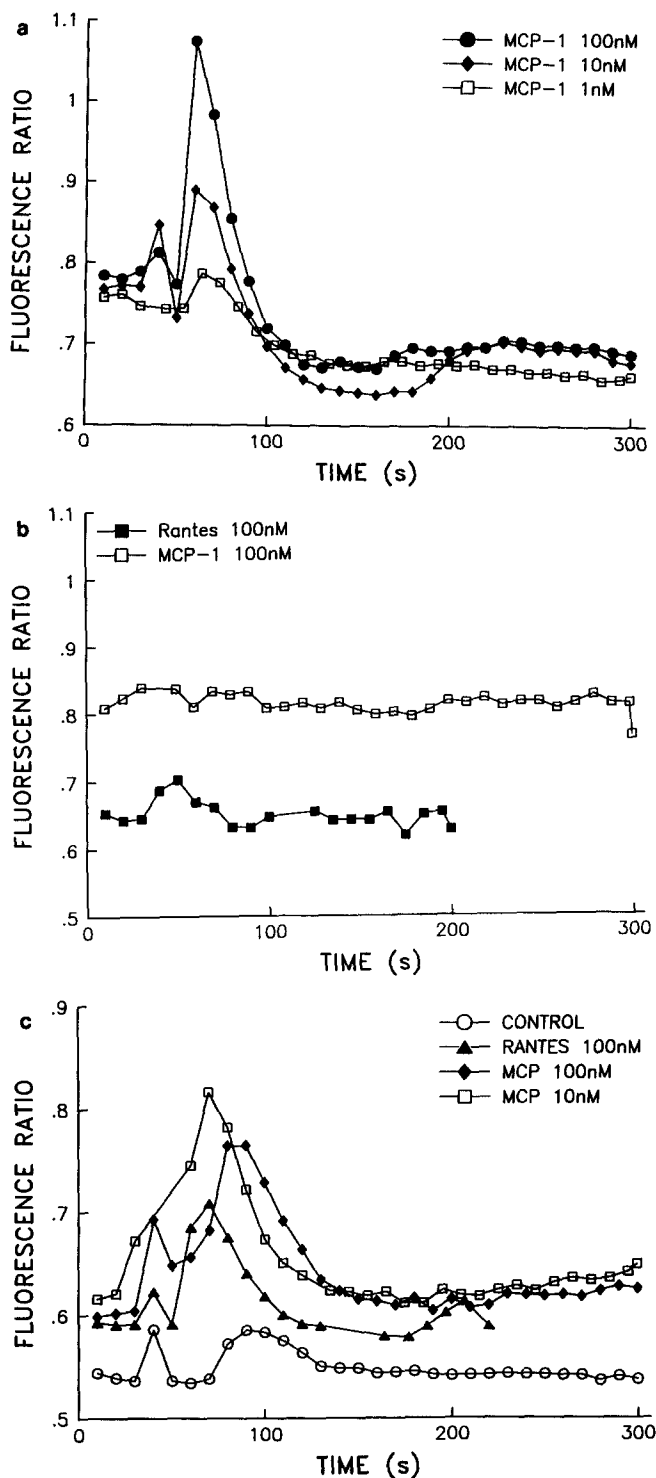


Figure 5. (a) MCP-1 dose-dependent ligand induced calcium flux measured by changes in Indo-1 fluorescence in THP-1 cells. No flux was seen with 100 nM Rantes. (b) Neither MCP-1 nor Rantes induced a calcium flux in Indo-1-loaded rabbit monocytes. (c) Dog monocytes undergo a ligand-induced calcium flux in response to both Rantes and MCP-1.

Discussion

These data provide the first published evidence for the presence of a shared receptor on THP-1 cells with high affinity for Rantes (K_d , 0.39 nM) and low affinity for MCP-1 (K_d , 35 nM). In addition these cells have a specific high affinity receptor (K_d , 0.19 nM) for MCP-1 alone, that in our hands has ~ 10 -fold higher affinity than that previously observed (17, 18). These high affinity receptors are likely regulated by G proteins as ligand binding is abolished by preincubation of membranes with GTP γ S with a nanomolar IC_{50} . Since the Rantes receptor shows oligospecificity in its binding of ligands, and as both Rantes and MCP-1 can be produced in nanomolar quantities at sites of chronic inflammation, it seems that some redundancy and biological complexity could be expected. Despite this, the Rantes receptor functionally should bind Rantes rather than MCP-1 under physiological conditions, because of its almost 100-fold greater affinity for Rantes than for MCP-1.

Scatchard analysis of the Rantes binding site shows a single high affinity site on intact cells. In addition, these are the first data showing the preservation of a C-C chemokine receptor on isolated membranes. Equilibrium binding of Rantes to isolated THP-1 cell membranes identified a single high affinity site on such membranes, but the binding affinity had been increased. The cause of the change in affinity upon cell disruption remains uncertain but may relate to alterations of G protein coupling, as receptor affinity is tightly regulated by G proteins and the majority of binding is lost upon

GTP γ S preincubation. Although MCP-1 binding can be demonstrated on isolated THP-1 membranes, no similar increase in affinity is seen in such membrane preparations (data not shown). Increases in affinity upon disruption of cells have been documented for the C5a receptor (15).

Binding of ligand to the Rantes receptor leads to chemotaxis of primary human monocytes in the absence of a measurable intracellular calcium flux. Binding of up to 100 nM Rantes to either human monocytes or THP-1 cells caused no calcium flux, although dog monocytes did show a Rantes-elicited calcium flux. This confirms the notion that a measurable generalized cytosolic calcium flux is not an essential prerequisite for chemotaxis. This does not, however, exclude the possibility that localized, strategic calcium fluxes below the resolution of the FACS[®] technique play a role in the transduction events necessary for chemotaxis. It is interesting to note that two highly related cytokine ligands, both acting upon G-linked receptors that should also exhibit significant homology, are so distinct in their postreceptor signaling pathways, namely MCP-1 being coupled to a Ca^{2+} flux, whereas Rantes elicited no such response. Assessing the biological relevance of such different pathways may shed light on the mechanisms by which monocytes discern their migratory path using the complex mixtures and potential redundancies of chemoattractant cytokines being generated at inflammatory sites.

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Address correspondence to Hugh Rosen, Department of Biochemical & Molecular Pathology, Building R80N-A26, Merck & Co., Inc., P.O. Box 2000, Rahway, NJ 07065.

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