Identification of RANTES Receptors on Human Monocytic Cells: Competition for Binding and Desensitization by Homologous Chemotactic Cytokines

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Summary

RANTES (regulated on activation, normal T expressed and secreted) is a member of the chemotactic cytokine (chemokine) β subfamily. High affinity receptors for RANTES have been identified on a human monocytic leukemia cell line THP-1, which responded to RANTES in chemotaxis and calcium mobilization assays. Steady-state binding data analyses revealed approximately 700 binding sites/cell on THP-1 cells with a Kd value of 400 pM, comparable to that expressed on human peripheral blood monocytes. The RANTES binding to monocytic cells was competed for by monocyte chemotactic and activating factor (MCAF) and macrophage inflammatory protein 1 (MIP-1) or, two other chemokine β cytokines. Although MCAF and MIP-1α competed for RANTES binding to monocytes with apparent lower affinity (with estimated Kd of 6 and 1.6, nM respectively) both of these cytokines effectively desensitized the calcium mobilization induced by RANTES. The chemotactic response of THP-1 cells to RANTES was also markedly inhibited by preincubation with MCAF or MIP-1α. In contrast, RANTES did not desensitize the THP-1 calcium mobilization and chemotaxis in response to MCAF or MIP-1α. These results, together with our previous observations that RANTES did not compete for MCAF or MIP-1α binding on monocytic cells, indicate the expression of promiscuous receptors on monocytes that recognize one or more cytokines within the chemokine β family.

A number of cytokines in the 8–11-kD molecular mass range have recently been identified that selectively chemoattract leukocyte subpopulations in vitro and elicit the accumulation of inflammatory cells in vivo. These cytokines belong to a superfamily of platelet 4 (PF4)-related molecules now known as chemokines (1). The chemokines are further divided into two subsets characterized by the positioning of the first two closely paired cysteines. Members of the chemokine α subfamily, represented by IL-8/neutrophil activating protein 1 (NAP-1) have an intervening amino acid between the first two cysteines (C-X-C) and are mainly neutrophil targeted (1), whereas members of the chemokine β subfamily, represented by monocyte chemotactic and activating factor (MCAF)/monocyte chemotactic protein 1 (MCP-1), have a C-C configuration and chemoattract mononuclear cells (1, 2). RANTES (regulated on activation, normal T expressed and secreted) (2) is a peptide that belongs to the chemokine β subfamily and was originally cloned from a subtracted cDNA library made from RNA isolated from a functional, nontransformed, antigen-stimulated T cell line (3). The recombinant RANTES protein is a highly basic, 8-kD polypeptide that chemoattracts human peripheral blood monocytes and T lymphocytes of the CD4+ /CD45 RO+ phenotype in vitro (4). RANTES has also been reported to rapidly stimulate Ca2+ mobilization and histamine release in human basophils and act as a chemoattractant for human eosinophils (5, 6).

In this study, we investigated the specific receptors for RANTES and explored the functional interrelationships between the binding and functional effects of recombinant human (rh) RANTES and other chemokine β cytokines on monocytic cells. We demonstrate that both human MCAF and macrophage inflammatory protein 1α (MIP-1α) were capable of competing for 125-I-RANTES binding to mono-

1 Abbreviations used in this paper: MCAF, monocyte chemotactic and activating factor; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α; NAP-1, neutrophil activating protein 1; RANTES, regulated on activation, normal T expressed and secreted; rh, recombinant human.
cytic cells. Preincubation with MCAF and MIP-1α inhibited the monocyctic cell response to RANTES in both Ca2+ mobilization and chemotaxis assays. The results suggest the expression of a RANTES receptor species on monocyctic cells which binds and transduces signals in response to several members of the chemokine β subfamily.

Materials and Methods

Cytokines. rhRANTES and rhMCAF were purchased from PeproTech Inc. and MIP-1α from R & D Systems Inc. Two radiolabeled rhRANTES preparations with the [125I] located on either histidine or tyrosine residues were kindly provided as a gift from Dr. M. Tsang (R & D Systems Inc., Minneapolis, MN) and Dr. B. Brown (Fuji Film, Tokyo, Japan) with an isoosmotic Percoll (Pharmacia, Uppsala, Sweden) gradient as described elsewhere (7). The monocytic cell preparations were always >90% pure as assessed by morphological criteria.

Chemotaxis Assay. Cell migration was evaluated using a 48-well microchemotaxis chamber technique (8). A 25-μl aliquot of testing reagents diluted in RPMI 1640 containing 1 mg/ml BSA was placed in the lower compartment, and 50 μl of cell suspension (1.5 × 10^6/ml monocytes or 4 × 10^6/ml THP-1 cells) was placed in the upper compartment of the chamber. The two compartments were separated by the polycarbonate filter (5-μm pore size; Nuclepore Corp., Pleasanton, CA). After incubation at 37°C (90 min for monocytes and 120 min for THP-1 cells) in air with 5% CO2, the filter was removed, fixed, and stained with Diff-Quik (Harleco, Gibbstown, NJ), and the number of migrating cells in five high-powered fields were counted. The mean (±SD) from one of three representative experiments are presented. The statistical significance of the number of cells migrating in response to stimuli vs. baseline (migration toward control medium) was calculated using Dunnnett’s test.

Binding of [125I]RANTES to Human Cells. In steady-state binding assays, 2 × 10^6 cells were incubated in duplicate with increasing concentrations of the test substance in a modified binding medium (RPMI 1640 containing 1 mg/ml BSA and 25 mM Hepes, pH 7.4) in a total volume of 200 μl. The nonspecific binding was determined by parallel incubation in the presence of 100-fold excess of unlabeled RANTES. After incubation at 4°C for 4 h, the cells were pelleted through a 10% sucrose/PBS cushion. The tips of the tubes containing cells were cut and radioactivity was determined in a gamma counter (model 4000; Beckman Instruments, Inc., Fullerton, CA). The residual nonspecific bound radioactivity associated with cells in the presence of unlabeled RANTES was subtracted from total bound radioactivity to yield specific binding. For estimation of binding sites/cell and Kd value, a nonlinear regression calculation described by Bates and Watts (9) was used. The advantage of this method is to minimize the difference between the model and the available data in the least-squares sense.

In all cases, complete data generated in the assay was used in the regression analyses. Scatchard plots were reformatted presentation of the nonlinear regression. Although some of our data might hint at a more complex model (10), no significant improvement was achieved using models other than “one receptor species” model at the present time. The displacement curves of [125I]RANTES binding to monocyctic cells by different cytokines were generated by incubating cells with a constant concentration of [125I]RANTES for 4 h at 4°C in the presence of increasing amounts of unlabeled ligands. The cells were then pelleted through a sucrose cushion and the residual radioactivity determined.

Analysis of Intracellular Calcium Mobilization. Analysis of the changes in intracellular Ca2+ concentration ([Ca2+]i) of THP-1 cells was carried out using a photometer (Deltascan; Photon Technologies International, Inc., Princeton, NJ) as described (11). Briefly, THP-1 cells were incubated at a density of 10^6/ml for 30 min at 37°C in medium containing 5 μM Indo-1 AM (Molecular Probes, Inc., Eugene, OR). After 30 min, fresh medium was added diluting the cells to 5 × 10^6/ml, and the cells were further incubated for 30 min at 37°C. Cells were washed once with medium after loading with Indo-1, and held at room temperature in the dark until analysis. The [Ca2+]i was monitored with the Indo-1 loaded cells suspended at 37°C in Dulbecco’s PBS with Ca2+ and Mg2+ supplemented with 5 mM glucose. Indo-1 excitation was at 358 nm with detection of bound dye at 402 nm (violet) and free dye at 486 (blue).

Results

RANTES has previously been reported to be chemotactic for human peripheral blood monocytes and T lymphocytes (4). We obtained monocyte chemotactic responses over a similar dose range using commercially available rhRANTES. We subsequently examined the capacity of RANTES to activate the monocyctic cell line THP-1. THP-1 cells were derived from an acute monocyctic leukemia patient and have rare chromosomal abnormality (12). THP-1 cells were cultured in suspension and express abundant receptor sites for both MCAF and MIP-1α (13). In preliminary experiments, we observed that THP-1 cells, even while undergoing exponential growth, migrated through polycarbonate filters in response to a variety of chemotactic agents including bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Immunochemicals, St. Louis, MO), MCAF, and MIP-1α, but not to IL-8, which preferentially chemoattracts neutrophils and is a chemokine α subfamily member. As shown in Fig. 1, rhRANTES also induced THP-1 cell migration in a dose-dependent manner with a typical bell-shaped curve. The maximal induction of THP-1 cell migration by rhRANTES was obtained with 6–60 nM of the cytokine (Fig. 1).

The chemotactic rather than chemokinetic nature of THP-1 cell migration to rhRANTES was confirmed by checkerboard analysis (Table 1) in which the maximal induction of THP-1 cell migration by rhRANTES occurred always in the presence of a positive cytokine concentration gradient between the upper and lower wells of the chemotaxis chamber (higher concentration below the filter). In the presence of negative gradients, no directional migration was observed. At identical concentrations above and below the filter, little enhance-

700 Identification of RANTES Receptors on Human Monocytic Cells
Figure 1. Chemotactic response of THP-1 cells induced by rhRANTES and related cytokines. The chemotaxis assays were performed as described in the Materials and Methods. The results are from one representative experiment out of five performed. SD values were <15% of each mean value and were not plotted.

We next examined the presence of specific receptors for rhRANTES on monocytic cells. The binding capacity of 125I-RANTES by THP-1 cells showed a temperature dependence with higher (fourfold) ligand association at 37°C than at 4°C (Fig. 2). An intermediate ligand association with cells was observed at 22°C. Binding at 37°C and 22°C reached a maximum at 20 min (Fig. 2) and was associated with internalization of the ligand–receptor complex (data not shown). Assays performed at 4°C lead to specific ligand binding to the cell membrane since most of the bound ligand could be eluted from the cells by a brief exposure of the cells to 50 mM glycine-HCl (pH 3; data not shown). Maximal binding at 4°C without internalization was obtained after 4 h at 4°C. Consequently the receptor number and the affinity for RANTES were determined.

The binding isotherms of THP-1 cells (Fig. 3a), as well as peripheral blood monocytes (Fig. 3b) for 125I-RANTES was determined. Steady-state binding data analyses revealed ~700 high-affinity binding sites/cell for RANTES on THP-1 cells with a $K_d$ value of 400 pM. Similar receptor numbers and $K_d$ values were detected on peripheral blood monocytes. Human peripheral blood neutrophils that do not migrate to RANTES failed to show any specific binding sites for RANTES (data not shown). Thus, the presence of specific cell surface receptors for RANTES on monocytes directly correlates with their chemotactic reactivity to this chemokine.

The ligand specificity of RANTES receptors on monocytic cells was investigated in competition studies using various unlabeled ligands including several members of the chemokine superfamily. Cytokines including IL-1α, TNF, G-CSF, GM-CSF, MCSF, and the bacterial peptide fMLP, as well as chemokine α (C-X-C) cytokines such as IL-8 and

Table 1. Checkerboard Analysis of RANTES Chemotactic Activity for THP-1 Cells

| RANTES in lower wells | 0 | 0.12 | 1 | 2 | 6 | 12 |
|----------------------|---|------|---|---|---|----|
| nM                   |   |      |   |   |   |    |
| 0                    | 58 ± 7 | 81 ± 5 | 104 ± 14* | 155 ± 17* | 163 ± 11* |
| 0.12                 | 52 ± 3 | 59 ± 8 | 76 ± 8 | 104 ± 12* | 155 ± 9* |
| 1.2                  | 46 ± 8 | 53 ± 4 | 61 ± 8 | 89 ± 11 | 121 ± 13* |
| 6                    | 42 ± 5 | 39 ± 6 | 48 ± 7 | 57 ± 12 | 78 ± 6 |
| 12                   | 33 ± 7 | 46 ± 8 | 49 ± 10 | 59 ± 11 | 67 ± 4 |

Different concentrations of rhRANTES were placed in the upper and/or lower wells of the chemotaxis chamber. THP-1 cells (50 μl at 4 × 10⁶/ml) were placed in the upper wells. The chemotaxis assay was performed as described in Materials and Methods. Results are representative of three experiments performed.

* $p < 0.05$ vs control (with medium in the upper and lower wells)
Figure 3. Steady-state binding of \(^{125}\text{I}\)-RANTES to monocytic cells 2 x 10^6 THP-1 cells (A) or Percoll-purified peripheral blood monocytes (B) in 200 µl binding medium were incubated at 4°C for 4 h with increasing concentrations of \(^{125}\text{I}\)-RANTES. The parallel duplicate tubes contained 100-fold excess of unlabeled rhRANTES and were assayed as described in Materials and Methods (Insets). Reformatted Scatchard plots of the non-linear regression calculation

Figure 4. Displacement of \(^{125}\text{I}\)-RANTES binding to THP-1 cells by unlabeled RANTES, MCAF, and MIP-1α. Duplicate samples of 2 x 10^6 THP-1 cells in 200 µl binding medium were incubated with 0.1 nM \(^{125}\text{I}\)-RANTES in the presence of increasing quantities of unlabeled cytokines. After incubation at 4°C for 4 h, the cells were centrifuged through sucrose cushion and the radioactivity in cell pellets was measured. Five experiments were performed with similar results.

Discussion

RANTES has previously been shown to induce in vitro chemotaxis in monocytes and CD45^+ T cells (4) and is a member of the chemokine β subfamily. We have confirmed and extended these observations using commercially available rhRANTES to induce in vitro chemotaxis of human peripheral blood monocytes and the human acute leukemia melanoma growth stimulating activity, did not compete for RANTES binding to monocytic cells (data not shown). In contrast, rhMCAF and MIP-1α, two members of the chemokine β (C-C) subfamily, were capable of competing for RANTES binding to THP-1 cells (Fig. 4) as well as on monocytes (data not shown). The displacement curves show that unlabeled rhRANTES competed for \(^{125}\text{I}\)-RANTES with a similar affinity as determined in the steady-state binding assays (300 vs. 400 pM). rhMCAF competed for RANTES binding with apparent lower affinity (about 6 nM), whereas rhMIP-1α (about 6 nM), whereas rhMIP-1α competed for RANTES receptors with an intermediate affinity (about 1.6 nM).

We next performed crossdesensitization experiments in calcium mobilization and chemotaxis assays to establish the functional significance of RANTES receptor utilization by MCAF and MIP-1α. Transient increases in cytosolic free calcium is an early event in signal transduction during leukocyte activation with chemotactic agonists (14-17). As shown in Fig. 5, all recombinant chemotactic cytokines for THP-1 cells induced rapid calcium mobilization in these cells, although RANTES was less potent as compared with the same concentration of MCAF or MIP-1α (Fig. 5 A). Prior incubation of the cells with maximally effective doses of RANTES (60 nM) did not prevent the subsequent response to 12 nM MCAF (Fig. 5 B) or MIP-1α (Fig. 5 C). In contrast, the subsequent response to 12 nM rhRANTES was abolished when the cells were initially stimulated with 60 nM MCAF (Fig. 5 B) or MIP-1α (Fig. 5 C). FMLP did not show any crossdesensitizing effect on the RANTES response (data not shown). The unidirectional desensitization of rhRANTES by rhMCAF or rhMIP-1α was also observed in chemotaxis assays (Table 2). Precubation of THP-1 cells for 30 min at 37°C with 30 nM rhRANTES did not markedly reduce the cell migration in response to either 6 nM rhMCAF or MIP-1α. However, preincubation of cells with 30 nM rhMCAF or MIP-1α markedly inhibited the cell migration to rhRANTES.
cell line THP-1. In addition to being chemotactic for monocytes, rhRANTES also induced calcium mobilization in THP-1 cells. We therefore utilized this cell line as a more homogeneous cell source to further characterize binding of 125I-RANTES to cell surface receptors.

Our initial experimental attempts to identify RANTES receptors at 4°C with conventional binding medium that was previously used to characterize IL-8 (18) and MIP-1α or MIP-1β (13) receptors were not successful. Increasing concentrations of unlabeled rhRANTES paradoxically yielded increased adsorption of radioactivity to cells. By optimizing binding conditions using a lower concentration of the carrier protein BSA (1 mg/ml) and slightly higher pH (pH 7.4), we detected specific competitive binding of 125I-RANTES on THP-1 cells, as well as on peripheral blood monocytes. Steady-state binding data analyses revealed a single class of high-affinity receptors on THP-1 cells and human peripheral blood monocytes with ~700 binding sites/cell and a $K_d$ value of 400 pM. The requirement for lower carrier protein concentration and higher pH may be based on a reduction in nonspecific aggregate formation by this highly basic peptide cytokine, as has been seen for other ligands for the chemokine $\beta$ family (19).

Of the many unlabeled ligands tested, only MCAF and MIP-1α, two members of the chemokine $\beta$ subfamily, competed for 125I-RANTES binding to THP-1 cells. Although the affinity of MCAF and MIP-1α for RANTES receptor is lower (6 and 1.6 nM, respectively) when present in excess, both MCAF and MIP-1α effectively blocked 125I-RANTES

![Figure 5. Desensitization of RANTES induced Ca$^{2+}$ mobilization by MCAF and MIP-1α](image)

Table 2. Desensitization of rhRANTES Chemotactic Activity for THP-1 Cells

| Pretreatment of cells | Number of migrated cells (% reduction*) |
|----------------------|----------------------------------------|
|                      | Medium       | RANTES       | MCAF         | MIP-1α        |
| Medium               | 67 ± 5       | 220 ± 23     | 275 ± 29     | 254 ± 31      |
| RANTES               | 82 ± 11      | 105 ± 6 (75)*| 264 ± 18 (5) | 240 ± 19 (7)  |
| MCAF                 | 79 ± 12      | 118 ± 7 (66)*| 122 ± 12 (72)*| 203 ± 21 (27)*|
| MIP-1α               | 84 ± 6       | 122 ± 12 (64)*| 236 ± 31 (19)*| 119 ± 17 (72)*|

THP-1 cells (5 x 10^6/ml) were incubated with assay medium (control) or different cytokines (at 200 ng/ml) for 30 min at 37°C, washed, then resuspended in assay medium. 50 μl of cell suspension were placed in the upper wells of the chemotaxis chamber. The lower wells contained control medium or 50 ng/ml of cytokines. The assay was performed as described in Materials and Methods.

* Percent inhibition of chemotaxis was calculated with the formula $1 - \frac{\text{migration of treated cells to cytokines} - \text{medium control}}{\text{migration of untreated cells to cytokines} - \text{medium control}} \times 100/100$

* $p < 0.05$ compared with the migration of untreated cell to respective cytokines

703 Wang et al
binding to monocytic cells. To examine the functional significance of these binding results, we performed a series of desensitization (or deactivation) experiments. Pretreatment of THP-1 cells with MCAF or MIP-1α abolished the response to subsequent stimulation with RANTES in calcium mobilization assays. MCAF and MIP-1α also greatly reduced directional cell migration induced by RANTES. In contrast, prior treatment with RANTES did not have a significant effect on THP-1 cell responses to MCAF or MIP-1α in either calcium mobilization or chemotaxis assays. This unilateral desensitization was presumably based on the quantitative difference in receptor numbers for those chemokines on monocytes. The 700 RANTES receptors/cell were probably too few to desensitize responses of cells expressing 18,000 MCAF and 3,000 MIP-1α receptors per cell.

In a previous study we identified specific binding sites on both circulating monocytes and THP-1 cells for [32P]-MCAF and [32P]-MIP-1α, as well as a third species which apparently bound both MCAF and MIP-1α (13). This, together with the present observations, suggest the presence of at least three distinct receptors for chemokine β subfamily members on monocyte THP-1 cells: (a) a RANTES receptor that binds three different ligands, RANTES, MCAF, and MIP-1α; (b) a unique receptor for MIP-1α; and (c) a unique receptor that binds MCAF, but not RANTES or MIP-1α. The RANTES receptor expressed on monocyte cells therefore appears to be promiscuous in nature in that it binds multiple ligands (RANTES, MIP-1α, and MCAF). Similar binding characteristics have been observed for one of the two IL-8 receptors that interact with multiple ligands from the chemokine α subfamily (20–22). Although the present study suggests the binding of multiple ligands (RANTES, MCAF, and MIP-1α) to a single receptor for RANTES, it is possible that these homologous cytokines may bind a common receptor subunit which is competed for by one of the ligands, as in the case of IL-3 and GM-CSF binding (23, 24). Alternatively, these receptors may transduce signals through a common G protein whose quantity may be limiting; the ligand binding could be dependent on the association of this molecule with the receptor. This latter possibility is unlikely since treatment of monocyte cells with pertussis toxin, a G protein inhibitor, abolishes chemotaxis and calcium mobilization, but does not affect RANTES receptor binding or competition by MCAF and MIP-1α. We are currently cloning the receptor genes for these members of the chemokine β subfamily to gain additional insight into the molecular architecture responsible for ligand-specific and promiscuous binding phenomena.

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705  Wang et al