Amino-terminally Modified RANTES Analogues Demonstrate Differential Effects on RANTES Receptors*

(Received for publication, July 16, 1999, and in revised form, August 28, 1999)

Amanda E. I. Proudfoot‡§, Raphaëlle Buser‡, Fredéric Borlat‡, Sami Alouani‡, Dulce Soler§, Robin E. Offord‡, Jens-Michel Schröder**, Christine A. Power‡, and Timothy N. C. Wells‡

From the ‡Serono Pharmaceutical Research Institute, 14 Chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland, *LeukoSite Inc., Cambridge, Massachusetts 02142, the **Department of Dermatology and Allergology, University of Kiel, Kiel, Germany, and Department de Biochimie Médicale, Centre Medical Universitaire, Geneva 1228, Switzerland

Modification of the amino terminus of regulated on activated normal T-cell expressed (RANTES) has been shown to have a significant effect on biological activity and produces proteins with antagonist properties. Two amino-terminally modified RANTES proteins, Met-RANTES and aminooxypentane-RANTES (AOP-RANTES), exhibit differential inhibitory properties on both monocyte and eosinophil chemotaxis. We have investigated their binding properties as well as their ability to activate the RANTES receptors CCR1, CCR3, and CCR5 in cell lines overexpressing these receptors. We show that Met-RANTES has weak activity in eliciting a calcium response in Chinese hamster ovary cells expressing CCR1, CCR3, and CCR5, whereas AOP-RANTES has full agonist activity on CCR5 but is less effective on CCR3 and CCR1. Their ability to induce chemotaxis of the murine pre-B lymphoma cell line, L1.2, transfected with the same receptors, consolidates these results. Monocytes have detectable mRNA for CCR1, CCR2, CCR3, CCR4, and CCR5, and they respond to the ligands for these receptors in chemotaxis but not always in calcium mobilization. AOP-RANTES does not induce calcium mobilization in circulating monocytes but is able to do so as these cells acquire the macrophage phenotype, which coincides with a concomitant up-regulation of CCR5. We have also tested the ability of both modified proteins to induce chemotaxis of freshly isolated monocytes and eosinophils. Cells from most donors do not respond, but occasionally cells from a particular donor do respond, particularly to AOP-RANTES. We therefore hypothesize that the occasional activity of AOP-RANTES to induce leukocyte chemotaxis is due to donor variation of receptor expression.

The chemokine family is responsible for the trafficking of leukocytes to maintain a correctly functioning immune system (1–5). Members of both the α- and β-subclasses of chemokines may be expressed constitutively and have been shown to be responsible for leukocyte homing, whereas many others are inducible and have been shown to be up-regulated in inflammatory conditions, both in human disease as well as animal models of inflammation. Inflammatory chemokines include members of the MCP1 and MIP families, as well as the closely related proteins, RANTES and eotaxin.

Chemokines mediate their biological effects through seven transmembrane-spanning, G-protein-coupled receptors, which also serve as the coreceptor with CD4 for HIV-1 infection. Cellular migration is a consequence of several signaling events, and many intracellular changes such as actin polymerization, shape change, and receptor polarization are implicated (4). Many investigations have shown the importance of the amino terminal region of the chemokine receptors for receptor activation via G-protein-coupled signal transduction. Several members of the CXC family have a three-residue motif, ELR (Glu-Leu-Arg), preceding the first two cysteines that is essential for receptor binding and for neutrophil chemotactic activity. Truncation of the first 5 residues, so that the arginine preceding the CXC motif of interleukin-8 is retained, produces a receptor antagonist (5) although activity of this antagonist has not been reported in vivo. Similarly modification of the amino-terminal region in several CC chemokines produces proteins with antagonistic properties. Deletion of the first 8 residues of RANTES results in a protein that is unable to mobilize calcium or to induce chemotaxis and is able to antagonize the response of the ligands that bind to RANTES receptors (6). A similar truncation of MCP-1 also produces a protein antagonist when the recombinant protein is produced in Escherichia coli, produces a nanomolar potent antagonist of monocyte migration, Met-RANTES (9). Similarly the chemical coupling of a five-carbon alkyl chain to the oxidized amino-terminal serine results in AOP-RANTES which is a potent inhibitor of HIV-1 infection of CCR5-using strains (10). Such chemokine receptor antagonists have been shown to prevent cellular recruitment in several murine models of inflammation (11–14).

Ligand binding triggers phosphorylation of the carboxyl terminus of the receptor by serine/threonine kinases which leads to receptor endocytosis. Chemokine receptor endocytosis is independent of G-protein coupling (15). Met-RANTES is unable to induce significant endocytosis of CCR1 (16) and CCR5 (17), whereas AOP-RANTES has recently been shown to be more potent than RANTES in receptor activation resulting in CCR5 receptor endocytosis (17). Down-regulation from the cell surface of chemokine receptors that function as coreceptors for HIV infection has been proposed to be a key mechanism for the inhibitory effects of these chemokines on HIV-1 cell entry (15, 17).
We have further investigated the abilities of the modified chemokines Met-RANTES and AOP-RANTES to activate receptors via the G-protein-coupled pathways both in cell lines overexpressing recombinant receptors as well in primary leukocytes. Our results show that the ability of Met-RANTES or AOP-RANTES to elicit functional responses in eosinophils is donor-dependent and in monocytes is dependent on their differentiation into the macrophage phenotype. We therefore investigated their abilities to induce chemotaxis on cell lines transfected with each of the known RANTES receptors. In these systems AOP-RANTES is a full agonist for activation of CCR5-bearing cells but is weaker in activating CCR1 and CCR3. Met-RANTES has only weak activity on all three RANTES receptors.

EXPERIMENTAL PROCEDURES

Reagents— Unless otherwise stated, all chemicals were purchased from Sigma. Enzymes were from New England Biolabs, and chromatographic material was from Amersham Pharmacia Biotech. The anti-CCR5 mAb (clone MC-1) was a kind gift of Dr. Matthias Mack.

Recombinant Chemokines— RANTES and MIP-1α were purified as described (18). Met-RANTES was produced according to Ref. 9, and the chemical conjugate AOP-RANTES was produced as described (10). 125I-MIP-1α and 125I-MCP-3 were obtained from Amersham Pharmacia Biotech. MIP-1β, MCP-3, and eotaxin were purchased from PeproTech.

Stably Transfected Cell Lines Expressing CC Chemokine Receptors— CHO stably transfected cells with CCR1, CCR3, and CCR5 were produced as described (19). CCR1, CCR3, and CCR5 L1.2 cell transfecants were generated as described previously (20).

Competition Equilibrium Binding Assays— The affinities of Met-RANTES and AOP-RANTES for CCR1 and CCR3 was determined by a scintillation proximity assay (SPA) using membranes prepared from CHO cells expressing the appropriate CC chemokine receptor as described (19). Briefly, 2 μg of membrane were incubated with 0.1 nM 125I-MIP-1α or 0.1 nM 125I-MCP-3 for CCR3 and increasing concentrations of the unlabeled chemokine upon agitation for 4 h at room temperature.

Receptor Expression— mRNA for β-chemokine receptors CCR1, CCR2, CCR3, CCR4, and CCR5 in freshly isolated monocytes and eosinophils was detected by RT-PCR. Total RNA was isolated from eosinophils and monocytes using Trizol™ (Life Technologies, Inc.). Reverse transcriptase reactions were performed on 1 μg of RNA using an oligo(dT) primer with the Superscript™ preamplification system (Life Technologies, Inc.). One-twentieth of the reverse transcriptase reaction mixture was then subjected to 35 cycles of PCR (2 min at 94 °C; 2 min at 55 °C, and 2 min at 72 °C) using Amplitaq™ (Perkin-Elmer) in a 50-μl reaction mixture containing 50 pmol of sense and antisense primer pairs for the CC chemokine receptors CCR1, CCR2, CCR3, CCR4, and CCR5, and GAPDH as a control for the quality of the cDNA used in each PCR reaction, in an MJ Research DNA engine. Primers were designed to amplify the full coding sequence (~1.1 kb), based on the receptor sequences obtained from the GenBank™ data base. The predicted size of the GAPDH product was 1 kb. In addition, control PCR reactions were performed with each primer pair on RNA samples that had had the absence of reverse transcriptase (results not shown). The identity of PCR products migrating at the predicted size was verified following gel purification using a Wizard PCR preps kit (Promega), by direct sequencing using the same primers as for the PCR reaction, in an ABI 377 DNA sequencer.

Cell surface expression of CCR5 was determined by flow cytometry using the monoclonal anti-CCR5 antibody MC-1 as described (17).

RESULTS

Competition Equilibrium Binding Assays— The extension of the amino terminus of RANTES does not abolish the capacity to bind to the three RANTES receptors, CCR1, CCR3, and CCR5. We have previously reported that Met-RANTES retains its binding to CCR1 (9) and that both modified chemokines retain their high affinity binding to CCR5 (10). Although AOP-RANTES binds to CCR5 with classical monophasic binding properties, Met-RANTES and Met-RANTES display biphasic displacement of the tracer. We have now compared the affinity of RANTES, AOP-RANTES, and Met-RANTES for CCR1 and CCR3 in a membrane-based scintillation proximity assay (SPA) shown in Fig. 1. AOP-RANTES has a comparable affinity to RANTES on CCR1, displaying an IC50 of 2 nM compared with 1.7 nM for RANTES, whereas Met-RANTES has a lower affinity with an IC50 of 30 nM as previously reported (9). For CCR3,
using 125I-MCP-3 as tracer to avoid the difficulties encountered using RANTES as tracer (23), we were not able to obtain the high affinity reported by Daugherty et al. (24) but obtained similar results to those reported by Ponath et al. (20) using heterologous competition. RANTES showed an IC50 value of 100 nM, and consistent with CCR1, Met-RANTES had a lower affinity with an IC50 value of 32 nM. AOP-RANTES showed a higher affinity with an IC50 value of 3.2 nM.

**mRNA Receptor Expression in Monocytes and Eosinophils**—mRNA for CCR1, CCR2, CCR3, CCR4, and CCR5 was analyzed in monocytes and eosinophils by RT-PCR. mRNA was detected for the five receptors in monocytes from three separate donors (Fig. 2A). However, in eosinophils from a hyper eosinophilic, but non-atopic patient, only CCR3 and CCR1 were detected (Fig. 2B) as has been previously reported (20, 24), whereas in eosinophils from an allergic patient, mRNA for receptors CCR1, CCR2, CCR3, CCR4, and CCR5 was detected (Fig. 2C).

**Ligand-induced Monocyte Responses**—The migration of freshly isolated monocytes in response to five chemokines was tested as follows: RANTES for CCR1, CCR3, and CCR5 activity; MCP-1 for CCR2; eotaxin for CCR3; TARC for CCR4, and MIP-1β for CCR5, in order to investigate functional receptor expression. Monocytes from four donors tested responded to these ligands in separate experiments (Fig. 3A). The migration was greatest in response to RANTES and MCP-1, showing chemotaxis indices of 5 and 7, respectively, whereas the CCR3-, CCR4-, and CCR5-mediated responses induced by their respective ligands resulted in lower efficacies ranging between 3 and 4.

Monocytes prepared from all donors showed a robust calcium mobilization in response to RANTES and MCP-1 in accordance with their efficacy in inducing monocyte migration, whereas eotaxin, TARC, and MIP-1β were unable to elicit a response (Fig. 3B). Met-RANTES and AOP-RANTES were unable to stimulate calcium mobilization in freshly isolated monocytes from all donors (results not shown). However, after 24 h in culture, AOP-RANTES was able to induce a robust calcium response in a dose-related manner in monocytes, and this response was significantly greater after 48 h in culture (Fig. 3B). Met-RANTES was not tested in view of its weak activity on all RANTES receptors.

**Antagonism of Chemotaxis**—The ability of Met-RANTES and AOP-RANTES to antagonize chemotaxis was investigated against ligands with different receptor usage. Both proteins were able to antagonize RANTES-induced chemotaxis of freshly isolated monocytes. Met-RANTES inhibited RANTES-induced monocyte chemotaxis with an IC50 of 11 nM, whereas AOP-RANTES was more potent with an IC50 of 1.4 nM (Fig. 4A).

Met-RANTES was inefficient in inhibiting the response induced by ligands which are specific for CCR3. Inhibition of eotaxin-induced monocyte chemotaxis was only observed at high concentrations showing an IC50 of 150 nM (Fig. 4B), and it was almost ineffective at antagonizing the eotaxin chemotaxis induced by eotaxin, although it was able to efficiently inhibit eosinophil chemotaxis induced by RANTES with an IC50 of 30 nM (Fig. 5). However, it was able to weakly inhibit MCP-3-induced eosinophil chemotaxis with an IC50 of 330 nM. AOP-RANTES, in accordance with its greater affinity for CCR3 than Met-RANTES, was able to inhibit eotaxin-induced monocyte chemotaxis (Fig. 4B) with nanomolar potency (IC50 of 1 nM) and eotaxin-induced eosinophil chemotaxis with an IC50 of 45 nM (Fig. 5).

In accordance with the retention of high affinity binding of both Met-RANTES and AOP-RANTES for CCR5, they were both able to efficiently inhibit MIP-1α-induced monocyte chemotaxis with IC50 values of 1.0 and 2.3 nM, respectively (Fig. 4C).

**Leukocyte Responses to Met-RANTES and AOP-RANTES**—The ability of Met-RANTES and AOP-RANTES to induce monocyte chemotaxis was investigated with cells isolated from 6 and 3 donors, respectively, in comparison to the chemotaxis induced by RANTES (Fig. 6, A and B). The chemotaxis index observed with RANTES varied from 4 to 11 depending on the donor. No response was observed for both modified proteins in almost all of the donors tested, with the exception of one donor whose monocytes responded only to AOP-RANTES with a chemotaxis index of 3.7, compared with 6 for RANTES using cells from the same donor in the same experiment.

Eosinophil chemotaxis was similarly tested from several donors. Of the 8 donors tested, the eosinophils from 3 donors did not respond to Met-RANTES (Fig. 6C). The remaining 6 patients showed a small response that did not exceed 20% of the response induced by RANTES on the eosinophils from the same donor. Similarly, AOP-RANTES showed no activity on eosinophils from one donor out of the four tested, whereas it induced a small response in two others. However, in the fourth donor, AOP-RANTES induced a chemotactic response equivalent to that induced by RANTES (Fig. 6D).

**Receptor Activation in Recombinant Transfected Cell Lines**—Both Met-RANTES and AOP-RANTES tested at 100 nM were able to induce a calcium response in CHO cells expressing CCR1, CCR3, and CCR5 (Fig. 7A). However, the kinetics of the response elicited by the modified proteins was significantly slower than that elicited by RANTES, with the exception of AOP-RANTES on the CHO/CCR5 cell line. Chemokines generally elicit a robust response within 2–4 s, whereas the response elicited by the amino-terminally modified proteins required 8–10 s (Fig. 7B). It is therefore difficult to judge whether the concentration of calcium mobilized reflects the actual activity of these modified proteins measured in this assay or is in fact an overestimate.

The ability of Met-RANTES and AOP-RANTES to elicit cellular migration through the three RANTES receptors was investigated using the murine pre-B cell line L1.2, transfected with the individual receptors (Fig. 8). Met-RANTES was inactive on CCR1 and CCR3 but showed weak activity on CCR5 with significantly reduced potency. AOP-RANTES was able to induce a response comparable to wild type RANTES through CCR5 (Fig. 8C). Furthermore, this derivative was more potent than Met-RANTES on the two other receptors, since it showed moderate efficacy on CCR3 (Fig. 8B), but less on CCR1 (Fig. 8A).

**Cell Surface CCR5 Expression on Differentiating Monocytes**—Cell surface expression was investigated for CCR5 on monocytes using the monoclonal antibody, MC-1. No CCR5 was detectable by flow cytometry on freshly isolated monocytes from four separate donors (data not shown). After 24 h in culture, as the cells became adherent, half the population...
showed surface CCR5 expression, whereas after 48 h, CCR5 surface expression was detectable on the entire monocyte-derived macrophage culture (Fig. 9a).

**DISCUSSION**

The modification of the amino terminus of RANTES by the addition of either a single amino acid due to the retention of the initiating methionine in recombinant RANTES (Met-RANTES) expressed in *E. coli* (9) or by the chemical coupling of a penta-carbon alkyl chain (AOP-RANTES) (10) results in proteins that are able to inhibit agonist-induced activities with nanomolar potency *in vitro*. AOP-RANTES has been shown to be the most potent inhibitor of HIV-1 infection mediated by CCR5 reported to date (10, 25). Met-RANTES has been shown to significantly

---

**Fig. 3.** Functional responses of monocytes to RANTES, MCP-1, eotaxin, TARC, and MIP-1β. a, Boyden chamber assays of chemotaxis induced by MCP-1, RANTES, eotaxin, TARC, and MIP-1β were performed as described in the text. The results shown are the average of four separate experiments with cells from four donors. b, calcium mobilization measured in Fura-2-AM-loaded monocytes. The results shown are a representative set from four separate experiments.
in the lower chamber. Met-RANTES inhibition of RANTES (10 nM agonist was incubated with the antagonist and AOP-RANTES. (Motaxis. The antagonists Met-RANTES (') and MCP-3 (') and MCP-1-β-induced chemotaxis. The antagonists Met-RANTES (●) and AOP-RANTES (●) were placed in the lower chamber, and the average of 3 experiments for eotaxin, 8 experiments for MIP-1β, and 10 experiments for RANTES are shown.

reduce the inflammatory symptoms in several animal models of inflammation including crescentic glomerular nephritis (11), rheumatoid arthritis (12), airways inflammation (26), and organ transplant rejection (14).

These proteins were initially described as being inactive in calcium mobilization and chemotaxis assays in the pro-monocytic cell line, THP-1 (9), and on freshly isolated monocytes (10). However they retain the ability to activate certain cellular responses. AOP-RANTES is more active than RANTES in inducing CCR5 internalization (17). Receptor endocytosis is known to be an agonist-mediated event involving G protein-coupled receptor kinase-mediated phosphorylation of the carboxyl-terminal region (27) but does not involve the classical G-protein-linked cascade (15). Another modified RANTES protein, which is produced by the truncation of the first 8 amino acids to form RANTES-(9–68), is also able to mediate receptor internalization (15) but, in contrast to AOP-RANTES, is reported to be devoid of the ability to induce calcium mobilization and chemotaxis. From these observations it can be concluded that modification of the amino terminus of RANTES disturbs its ability to fully activate certain signaling events, while not affecting other receptor activation states that lead to events such as receptor internalization.

We have shown here that both Met-RANTES and AOP-RANTES are in fact capable of mediating calcium mobilization in CHO cells overexpressing RANTES receptors. However, the efficacy of these modified ligands should be interpreted with caution due to the difference in the kinetics of the response. The natural ligands induce a rapid, robust response to attain a certain magnitude of calcium influx, whereas the amount of calcium influx reported for the modified proteins requires a 2–3-fold increase in time of response, with the exception of AOP-RANTES activation of CCR5. This untypical response induced by these modified proteins could therefore lead to an overestimation of the calcium mobilized from internal stores typical of chemokine activity and could be in part the result of the opening of membrane calcium channels through alternative signal transduction mechanisms.

Calcium mobilization induced by the modified RANTES proteins was not detectable in primary cells, but this could be attributed to the sensitivity of the method used. We have observed that calcium mobilization cannot be detected in freshly isolated monocytes in response to eotaxin, TARC, and MIP-1β, yet the mRNA for CCR3, -4, and -5 is detectable by RT-PCR. In contrast monocytes are able to respond to these ligands in the chemotaxis assays indicating that the appropriate receptors are expressed on the cell surface but possibly at levels too low to allow detectable calcium mobilization. RANTES and MCP-1, which induced a robust calcium response, were significantly more efficacious than eotaxin and TARC, which were not able to induce calcium, in mediating chemotaxis. Although freshly isolated monocytes had undetectable levels of CCR5 using the mAb MC-1, the specific CCR5 ligand MIP-1β was used to detect cell surface CCR5 expression by flow cytometry. Since RANTES is able to induce a robust calcium response in freshly isolated monocytes, presumably it does so through one of the other receptors, such as CCR1. It has been previously reported that circulating monocytes do not express detectable levels of surface CCR5 which is rapidly up-regulated as the cells differentiate into macrophages (28–30). This is particularly relevant for HIV infection since HIV viral particles are not detected in circulating monocytes (31), whereas resident tissue macrophages are thought to be one of first targets of the HIV virus during transmission (32).
By using L1.2 transfectants, we have shown that the modified proteins are able to activate the RANTES receptors CCR1, CCR3, and CCR5 to mediate chemotaxis with varying efficiency. Met-RANTES is the least active, showing only minimal activity on CCR5, whereas AOP-RANTES is very poor at activating CCR1, has half the activity of RANTES on CCR3, but is fully active on CCR5. However, AOP-RANTES is only on rare occasions able to mediate the chemotaxis of primary cells. Although monocytes do have functional CCR5 as shown by the ability of MIP-1β to induce chemotaxis of these cells, AOP-RANTES is unable to do so despite its full activity on the L1.2/CCR5 transfectants. The possibility remains that AOP-RANTES can only activate a certain conformer of CCR5, which has been recently shown to adopt multiple conformational states (33) and that MIP-1β is less sensitive to the different conformational states.

The differential expression of chemokine receptors has been addressed recently by several laboratories. Here we show by the occasional reactivity of certain leukocytes to AOP-RANTES in in vitro chemotaxis assays that β-chemokine receptor expression varies considerably from donor to donor and may depend on the health state of the individual. The up-regulation of certain chemokine receptors by cytokine treatment in vitro was first reported by Loetscher et al. (34) who showed that in T lymphocytes interleukin-2 was able to significantly affect the levels of CCR1 and -2. Similarly, human neutrophils do not respond to MIP-1α and eotaxin, but treatment with inflammatory stimuli such as interferon-γ leads to up-regulation of CCR1 and CCR3 rendering these cells responsive to their ligands (35). Differential chemokine receptor expression has also been noticed in eosinophils using a murine model of airways inflammation following sensitization with cockroach antigen.
In this model, circulating eosinophils express predominantly CCR3 and CCR1, whereas the eosinophils that have been recruited into the airways express CCR2, -4, and -5 in addition to CCR1 and CCR3. Although the expression of CCR5 on human eosinophils reported here was only detected on a single patient and is shown at the mRNA level, the reactivity of AOP-RANTES in a different allergic patient could indicate that CCR5 can be expressed in eosinophils in certain individuals. We are currently screening eosinophils from a panel of donors for CCR5 expression with anti-CCR5 mAbs.

Met-RANTES has been reported to prevent the recruitment of eosinophils into the airways following ovalbumin challenge in ovalbumin-sensitized mice (1, 26). In view of the fact that Met-RANTES has a reduced affinity for CCR3 and is also very poor at inhibiting human eotaxin-mediated chemotaxis, as well as murine eosinophil chemotaxis induced by eotaxin, it must mediate the inhibition of eosinophil recruitment by a different mechanism. Either the inhibition is mediated by another RANTES receptor such as CCR1, CCR5, or another as yet un-

known RANTES receptor or, alternatively, the effect is indirect. Since it also reduces the recruitment of lymphocytes in vivo (11), the production of specific eosinophil chemoattrac-
tants in situ may be modulated.

The potent antagonistic effects of Met-RANTES and AOP-RANTES in inhibiting inflammation and HIV infection, respectively, render them candidates as potential therapeutic agents. Here we have shown that both modified proteins have partial agonist activity and that AOP-RANTES is fully active on CCR5. These observations are important when considering their application therapeutically. However, a report of the systemic administration of recombinant fully active MIP-1α in a clinical trial showed no inflammatory side effects (37). We therefore conclude that the chemoattractant property of chemokines does not necessarily induce inflammation per se, but accessory signals may be required. This hypothesis is substantiated by data from transgenic mice engineered to overexpress a chemokine ligand such as MCP-1 (38). In conclusion, the data presented here must be taken into consideration when envisaging such a therapeutic strategy. We are currently investigating the effects of administration of higher doses of these modified chemokines than those reported to date in animal models of inflammation.

Acknowledgments—We thank M. Mack for the anti-CCR5 antibody, MC-1, and A. Meyer and B. Dufour for excellent technical assistance.

REFERENCES
1. Wells, T. N. C., Power, C. A., and Proudfoot, A. E. I. (1998) Trends Pharmacol. Sci. 19, 376–380
2. Luster, A. D. (1998) N. Engl. J. Med. 338, 436–445
