Inhibition of Airway Inflammation by Amino-Terminally Modified RANTES/CC Chemokine Ligand 5 Analogues Is Not Mediated through CCR3


*J Immunol* 2003; 171:5498-5506; doi: 10.4049/jimmunol.171.10.5498
http://www.jimmunol.org/content/171/10/5498

**References**
This article **cites 52 articles**, 28 of which you can access for free at:
http://www.jimmunol.org/content/171/10/5498.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inhibition of Airway Inflammation by Amino-Terminally Modified RANTES/CC Chemokine Ligand 5 Analogues Is Not Mediated through CCR3


Chemokines play a key role in the recruitment of activated CD4+ T cells and eosinophils into the lungs in animal models of airway inflammation. Inhibition of inflammation by N-terminally modified chemokines is well-documented in several models but is often reported with limited dose regimens. We have evaluated the effects of doses ranging from 10 ng to 100 µg of two CC chemokine receptor antagonists, Met-RANTES/CC chemokine ligand 5 (CCL5) and aminooxypentane-RANTES/CCL5, in preventing inflammation in the OVA-sensitized murine model of human asthma. In the human system, aminooxypentane-RANTES/CCL5 is a full agonist of CCR5, but in the murine system neither variant is able to induce cellular recruitment. Both antagonists showed an inverse bell-shaped inhibition of cellular infiltration into the airways and mucus production in the lungs following allergen provocation. The loss of inhibition at higher doses did not appear to be due to partial agonist activity because neither variant showed activity in recruiting cells into the peritoneal cavity at these doses. Surprisingly, neither was able to bind to the major CCR expressed on eosinophils, CCR3. However, significant inhibition of eosinophil recruitment was observed. Both analogues retained high affinity binding for murine CCR1 and murine CCR5. Their ability to antagonize CCR1 and CCR5 but not CCR3 was confirmed by their ability to prevent RANTES/CCL5 and macrophage inflammatory protein-1β/CCL4 recruitment in vitro and in vivo, while they had no effect on that induced by eotaxin/CCL11. These results suggest that CCR1 and/or CCR5 may be potential targets for asthma therapy. The Journal of Immunology, 2003, 171: 5498–5506.
of HIV-1 infectivity (21) and had increased affinity for human RANTES/CCL5 receptors (22). Met-RANTES/CCL5 has been shown to retain partial agonist activity in the human system, and aminooxypentane (AOP)-RANTES/CCL5 is in fact a full agonist of human CCR5 (22–24). Therefore, we were interested to compare the effects of these two modified RANTES/CCL5 analogues in a model of inflammation, and in particular, to address the question of whether AOP-RANTES/CCL5 would have more pronounced anti-inflammatory properties than Met-RANTES/CCL5 due to the increased affinity observed for human RANTES/CCL5 receptors, or whether its full agonist activity observed on human CCR5 would produce increased inflammation. In this study, we report that both exhibit a bell-shaped curve of inhibition of cellular recruitment into the lungs, a phenomenon described for many years for agonist activity (25), but to our knowledge this is the first demonstration of this type of response for an antagonist. Moreover, it appears that the inhibition of eosinophil recruitment is not mediated by the inhibition of the activity of eotaxin/CCL11 through CCR3, because in contrast to human CCR3, where AOP-RANTES/CCL5 has high affinity, but Met-RANTES/CCL5 displays lower affinity (22), neither is able to compete for eotaxin/CCL11 binding to CCR3. These results suggest that blockade of CCR3 is not necessarily the only approach to treat conditions such as allergic asthma, but that targeting CCR1 and CCR5 may also be effective.

Materials and Methods

Reagents

Met-RANTES/CCL5 was produced as previously described with the following modifications (17, 26). The inclusion bodies were dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM DTT and 6 M guanidine hydrochloride, heated at 60°C for 60 min to ensure monomerization, and then dialyzed extensively against 1% acetic acid. The recombinant Met-RANTES/CCL5 protein remained soluble while the contaminating proteins precipitated, and was recovered from the supernatant by centrifugation at 10,000 × g for 30 min, lyophilized, and then resuspended in 50 mM Tri-HCl buffer, pH 8.0, containing 1 mM DTT and 6 M guanidine hydrochloride at a concentration of 1 mg/ml. Renaturation was conducted as previously described (26). AOP-RANTES/CCL5 was produced as previously described (21). The endotoxin contents were measured using the Kinetic-QCL Limulus amoebocyte lysate kit (BioWhittaker, Walkersville, MD). The lyophilized proteins were dissolved in water and then diluted into sterile 0.9% NaCl to achieve the required concentration in 200 μl for administration into the mice. Chinese hamster ovary (CHO)-transfected cells expressing the murine receptors CCR1 and CCR5 as well as a human embryonic kidney (HEK) transfectant expressing murine CCR3 (mCCR) were generated as previously described (27).

Immunoassay and allergen challenge of mice

BALB/c mice (20–25 g) of either sex were immunized with 10 μg of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in 0.2 ml of aluminum hydroxide (alum) (Serva, Heidelberg, Germany), administered i.p. Fourteen days later, animals were injected with 0.01, 0.1, 1, 10, or 100 μg of Met-RANTES/CCL5 or 0.01, 0.1, 1, or 10 μg AOP-RANTES/CCL5 i.p. in the AOP-RANTES/CCL5-treated group, one group was treated with 80 μg i.p. of the neutralizing anti-IL-5 mAb TRFK-5 as a positive control for the inhibition of eosinophil accumulation in the airways. Control mice received an injection of 0.9% NaCl alone. In a parallel experiment, animals were treated with 0.001, 0.01, 0.1, 1, 10, or 100 μg of RANTES/CCL5. Thirty minutes later, mice were anesthetized by inhalation of 2% FORÈNE (isoflurane; Abbott, Cham, Switzerland) and 10 μg of OVA were administered to the lungs in a volume of 50 μl of 0.9% NaCl, via the intranasal route. Control mice were treated with 50 μl of 0.9% NaCl alone. This procedure was repeated daily for 5 days.

Quantification of cellular infiltration into the lung

Seventy two hours after the last Ag challenge, mice were sacrificed with i.p. injection of 14% urethane, the trachea cannulated, and bronchoalveolar lavage (BAL) performed by four repeated lavages with 0.3 ml of PBS injected into the lungs via the trachea. Total cell counts were performed with a hemocytometer after staining with trypan blue (Sigma-Aldrich) and cytospins were prepared (Shandon Scientific, Cheshire, U.K.). Slides were stained with Diff-Quik (Baxter Dade, Dudingen, Switzerland) and a differential count of 200 cells per slide was performed. The percentage of each leukocyte subtype was determined by counting their number under 400 magnification high power fields in oil immersion. To obtain the absolute number of each leukocyte subtype in the lavage, the percentages were multiplied by the total number of cells recovered from the BAL fluid.

Measurement of airway hyperresponsiveness in vivo

Bronchial hyperreactivity was measured by recording respiratory pressure curves by whole body plethysmography in response to inhaled methacholine (MCh; Sigma-Aldrich) using a Buxco apparatus (EMKA Technologies, Paris, France). The airway reactivity was expressed in enhanced pause (Penh) as described previously (28), where Penh is calculated as a value that corrects for airway resistance and turbulence, and intrapleural pressure in the same mouse using the following equation: Penh = (Te-Tr) – PePf/Pf, where Te = expiration time, Tr = relaxation time, PfPf = peak expiratory flow, and Pf = peak inspiratory flow.

Histology

Following the BAL, lungs were inflated with 0.6 ml of OCT (Tissue-tek; Miles, Elkhart, IN) and 20% sucrose (Sigma-Aldrich) (1:1 v/v). The lungs were then removed, snap-frozen, and 8–10-μm cryosections stained in methanol at 20°C for 2.5 min. Slides were stained with H&E (Fluka Chemie, Buchs, Switzerland) for gross morphology, diaminobenzidine substrate (Sigma-Aldrich) for cytidine-resistant peroxidase revealing the presence of eosinophils, and Alcian blue/periodic acid Schiff’s base (Fluka Chemika) for mucus production.

Lung-associated lymph node cellular composition analysis

Single cell suspensions were obtained from lung-associated lymph nodes after enzyme digestion with collagenase type IV (2.5 mg/ml) (Worthington Biochemistry, Lakewood, NJ) and 1% DNase I (Sigma-Aldrich). Cells were then washed and resuspended in RPMI 1640 medium. The cells were incubated with specific Abs to identify the cell types as follows: FITC-rat anti-mouse CD45 for leukocytes, FITC-rat anti-mouse B 220 for B lymphocytes, CyChrome-rat-anti-mouse-CD3, PE-rat-anti-mouse-CD4, FITC-rat anti-mouse-CD8, FITC-rat anti-mouse-CD69 for T lymphocytes, FITC-rat anti-mouse-F4/80 for macrophages, and FITC-hamster anti-mouse-CD11c for dendritic cells (all purchased from BD PharMingen (San Diego, CA), with the exception of rat anti-mouse F4/80 which was from Serotec (Oxford, U.K.)). Flow cytometry was performed after gating on the lymphocyte population using a FACSCalibur analytical flow cytometer (BD Biosciences, Mountain View, CA) and were analyzed using CellQuest software (BD Biosciences).

Equilibrium competition binding assays

The affinities of Met-RANTES/CCL5 and AOP-RANTES/CCL5 for mCCR1, mCCR3, and mCCR5 were determined by competition equilibrium binding assay in whole cells using CHO or HEK 293 cells transfected with the appropriate receptor. Briefly, 1.5×10^6 cells were incubated for 90 min at 20°C with 100 pM [125I]-labeled human RANTES/CCL5 for mCCR1, [125I]-labeled human eotaxin/CCL11 for mCCR3, and [125I]-labeled human macrophage inflammatory protein-1α (mMIP-1α) for mCCR5 and increasing concentrations of unlabeled chemokine in 100 μl of binding buffer, pH 7.2, containing 50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, and 0.5% BSA. After incubation, cells were washed three times with 200 μl of binding buffer containing 0.5 M NaCl under vacuum. Fifty microliters of scintillant were added and plates were counted with a beta scintillation counter (Wallac, Gaithersburg, MD) for 1 min per well. Binding parameters were determined with the Graft 3.01 software (Eriticus Software, Staines, U.K.) using the equation B = B0/(1 + (L/KD))5

Cell preparation and migration assays

Mouse spleens were macerated and passed through a 70-μM filter and erythrocytes were removed by lysis in hemolytic Gey’s solution. T lymphocytes were purified by negative selection using the CD4+ T Cell Isolation kit (Miltenyi Biotec, Bergisch, Germany) according to the manufacturer’s instructions. Eosinophils were enriched by negative selection from spleen of IL-5 transgenic mice (CBA/CaH-TnN) provided by Prof. C. Sanderson (Animal Resources Centre, Canning Vale, Australia) (29). Rat-anti-mouse B220, anti-CD4, anti-CD8, and anti-class II-coated magnetic beads were used to deplete B, T, and APCs and to enrich the single cell population using a FACSCalibur analytical flow cytometer (BD Biosciences, Biosciences, Mountain View, CA) and were analyzed using CellQuest software (BD Biosciences).
lower chambers with $10^{-8}$ M of chemokine, using eotaxin/CCL11 for eosinophils and RANTES/CCL5 or MIP-1β/CCL4 for T lymphocytes. A set of wells containing chemotaxis medium alone was included as a negative control. Fifty microliters of the cell solution were diluted in chemotaxis medium at a concentration of $3 \times 10^{5}$ cells/ml and were added to the upper chambers. The plates were incubated in 5% CO$_2$ at 37°C for 60 and 45 min for eosinophils and T cells, respectively, and the number of migrated cells was determined on the lower surface by IBAS image analyzer software (Kontron, Plaisir, France). Data were analyzed using GraphPad 3.01 software.

Peritoneal cellular recruitment

Cellular recruitment was induced by i.p. injection of female BALB/c mice (8–12 wk) with 10 µg of RANTES/CCL5 or varying amounts of Met-RANTES/CCL5 and AOP-RANTES/CCL5 diluted in 0.2 ml of sterile, LPS-free 0.9% NaCl. Inhibition experiments were performed by injecting 10 µg of the antagonist 30 min before the agonist chemokine, RANTES/CCL5, eotaxin/CCL11, or MIP-1β. Basal peritoneal cell numbers were measured by the administration of 0.2 ml of 0.9% NaCl. Sixteen hours later, mice were sacrificed by inhalation of CO$_2$. Peritoneal lavage was performed with three washes of 5 ml of sterile, ice-cold PBS, and the lavages were pooled for individual animals. Samples were centrifuged at 600 × g for 10 min, resuspended in a final volume of 1 ml, and total leukocytes elicited were counted with a Neubauer hemacytometer.

Data analysis

All results are expressed as mean ± SEM. The Student’s two-tailed paired $t$ test was used to determine statistical significance between groups of mice, values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)) were considered significant.

Results

N-terminally modified RANTES/CCL5 analogue treatment inhibits Ag-induced airway inflammation

Allergen exposure in OVA-sensitized and -challenged mice induced a marked cell infiltration with 20 ± 2.4 × 10$^5$ total cells, notably eosinophils (6.95 ± 1.4 × 10$^5$ cells) and lymphocytes (5.7 ± 1.6 × 10$^5$ cells) into the airways 72 h after the last challenge. In contrast, in saline-sensitized and -challenged mice, the baseline cell levels were 2.8 ± 0.03 × 10$^5$ cells where macrophages comprised >99% of the cellular composition of the BAL fluid. In preliminary experiments, three doses of Met-RANTES/CCL5 were tested, 500, 50, and 5 µg/mouse, given 30 min before each intranasal OVA challenge. No significant inhibitory activity of cellular recruitment into the BAL fluid was observed at the 50 and 500 µg doses, whereas 77% inhibition was seen at the 5-µg dose compared with the positive control group consisting of OVA sensitization and challenge, but treated with saline (data not shown). The effect of low doses was then examined, and significant inhibition of cellular recruitment was observed at doses of 4, 1, and 0.25 µg/mouse showing 79, 85, and 82% inhibition compared with the positive control group (data not shown). A more extensive dose response was therefore performed, with doses ranging from 100 to 0.01 µg (Fig. 1, a and b) which confirmed the trend of the two preliminary experiments. Pretreatment with Met-RANTES/CCL5 at doses of 0.1 and 1 µg/mouse significantly inhibited cellular recruitment by 59 and 57% of total cells (8.9 ± 2.2 × 10$^5$ and 9.2 ± 4.2 × 10$^5$; Fig. 1a) and in particular by reducing eosinophils by 74 and 67% (1.6 ± 0.3 × 10$^5$ and 0.2 ± 0.15 × 10$^5$ cells, respectively, Fig. 1b) into the airways as assessed by BAL fluid analysis. Although lymphocytes represented only a small proportion of cells present in BAL fluid of allergen-exposed mice, their number was also decreased by Met-RANTES/CCL5 treatment at 0.1 and 1 µg/mouse (1.14 ± 0.3 × 10$^5$ and 1.54 ± 0.04 × 10$^5$ cells, respectively, compared with 5.7 ± 1.6 × 10$^5$ cells present in positive control group; results not shown). The inhibition was lost at low doses as expected, but surprisingly, the treatment with higher doses of 10 and 100 µg were also ineffective.

We then examined a second N-terminally modified RANTES/CCL5 analog, AOP-RANTES/CCL5, because this analog has been described as having enhanced affinity for human RANTES/CCL5 receptors, as well as enhanced agonist properties on one of them, CCR5. In view of its possibly enhanced affinity, the dose range was restricted to 0.01–10 µg. However, AOP-RANTES/CCL5...
Histological analysis

Similarly, histological analysis demonstrated that allergen provocation was associated with a marked increase in the cellularity of the lung tissue as demonstrated in Fig. 2d which contained a large number of eosinophils (Fig. 2e) and an increase in mucus production (Fig. 2f) as compared with saline-exposed mice (Fig. 2, a–c). Treatment with the RANTES/CCL5 variants, as shown for the dose of 1 μg of Met-RANTES/CCL5, markedly reduced the cellular infiltrate, in particular the number of infiltrating eosinophils and the increased mucus-producing cells along the airway epithelium (Fig. 2, g–i) correlating processes occurring in the lung tissue and the findings in BAL fluid.

The same pattern was observed for treatment with AOP-RANTES/CCL5, where the inhibition of total cellular infiltrates, in particular eosinophils, as well as mucus production, was greatest at the dose of 0.1 μg with little inhibition observed at either the highest or lowest doses (results not shown).

Ag-induced airway hyperresponsiveness was not reduced

The inhibition of lung inflammation as measured by the cellular recruitment into the lungs did not have a significant effect on bronchial hyperreactivity (results not shown). The Penh value determined for the OVA-sensitized and -challenged group was 5.6, and in the AOP-RANTES/CCL5-treated group, a small, but nonsignificant decrease was observed at the 1 and 0.1 μg doses, giving Penh values of 3.3 and 3.2, respectively, comparable to the effect of TRFK-5, which also produced a small, nonsignificant decrease with a Penh value of 3.5. The administration of RANTES/CCL5 caused neither an increase nor a decrease in hyperreactivity.

Peritoneal cellular recruitment

Because we observed loss of inhibition at high doses of the antagonists, we investigated whether they had intrinsic agonist activity in vivo. To test this, we measured their ability to recruit cells into the peritoneal cavity. RANTES/CCL5 shows a dose-related activity when injected i.p., which reaches a plateau at 10 μg (30). At a dose of 10 μg, RANTES/CCL5 induces a 2- to 3-fold increase in cell number as shown in Fig. 3. However, neither Met-RANTES/CCL5 (Fig. 3a) nor AOP-RANTES/CCL5 (Fig. 3b) was able to induce cellular recruitment at the doses tested.

Equilibrium competition binding assays

We have analyzed the affinity of AOP-RANTES/CCL5 and Met-RANTES/CCL5 to bind to the murine RANTES/CCL5 receptors by their ability to compete for the ligands of these receptors in heterologous competition assays. As has been previously reported, human RANTES/CCL5 cross-reacts with the murine system (31). Using iodinated human RANTES/CCL5 in the mCCR1 binding assay, human RANTES/CCL5 had an IC50 of 5.2 nM in this assay.

FIGURE 2. Histological evaluation of lungs of mice after Met-RANTES/CCL5 treatment. On day 21, lung tissue was excised and cryosections were prepared from mice challenged with either NaCl (a–c) or OVA (d–f) or OVA plus Met-RANTES/CCL5 treatment (1 μg) (g–i). Serial sections were either stained with H&E (a, d, and g), cyanide-resistant peroxidase for the presence of parenchymal eosinophils (b, e, and h), or Alcian blue for mucus production (c, f, and i). Original magnifications, ×13 (a, d, and g); ×66 (b, c, e, f, h, and i).
Both N-terminally modified RANTES/CCL5 analogues retained high affinity for mCCR1 with IC<sub>50</sub> values of 4.3 nM for AOP-RANTES/CCL5 and 12.2 nM for Met-RANTES/CCL5 (Fig. 4a). Similarly, high affinity of the analogues was retained for mCCR5 compared with that of the human wild-type RANTES/CCL5 (Fig. 4c). In this assay, we used mMIP-1α as the radiolabeled ligand, which has been reported to have the highest affinity for mCCR5 (32), and mMIP-1α had an IC<sub>50</sub> of 0.12 nM. RANTES/CCL5 competed for mMIP-1α with an IC<sub>50</sub> of 15 nM, whereas AOP-RANTES/CCL5 had a slightly increased affinity with an IC<sub>50</sub> of 6 nM, and Met-RANTES/CCL5 had a slight decrease with an IC<sub>50</sub> of 30 nM. However, contrary to the human system, neither wild-type RANTES/CCL5 nor the analogues were able to displace iodinated eotaxin/CCL11 from mCCR3 (Fig. 4b).

**Inhibition of chemotaxis in vitro**

Neither variant was able to significantly inhibit eotaxin/CCL11-induced eosinophil chemotaxis purified from IL-5 transgenic mice (Fig. 5a). In contrast, both analogues had potent activity in the inhibition of T lymphocyte chemotaxis induced by RANTES/CCL5 (Fig. 5b), the ligand for CCR1 and CCR5, as well as the specific CCR5 ligand, MIP-1β (Fig. 5c). AOP-RANTES/CCL5 and Met-RANTES/CCL5 demonstrated IC<sub>50</sub> values of 0.7 and 4.3 nM, respectively, for the inhibition of RANTES/CCL5-induced recruitment. The IC<sub>50</sub> values for the inhibition of MIP-1β was 0.8 nM for AOP-RANTES/CCL5 and 5.8 nM for Met-RANTES/CCL5.

**Inhibition of cellular recruitment in vivo**

To confirm the ability of both N-terminally modified RANTES/ CCL5 analogues to antagonize RANTES/CCL5 receptor ligands as determined in vitro, we have further investigated their ability to inhibit these ligands in vivo by using a peritoneal cellular recruitment assay. The antagonists were given 30 min before the agonist challenge. Using this assay, we were able to confirm that both Met-RANTES/CCL5 (Fig. 6a) and AOP-RANTES/CCL5 (Fig. 6b) could inhibit RANTES/CCL5-mediated cellular recruitment as
well as that induced by MIP-1β/CCL4 (Fig. 6). However, neither analog was able to inhibit the recruitment induced by eotaxin/CCL11.

The cellular composition of lung-associated lymph nodes is modified by treatment

We observed that lung-associated lymph nodes were enlarged in OVA-sensitized and -challenged mice treated with the N-terminally modified analogues at the doses where cellular infiltration was observed. Therefore, the cellular composition of lung-associated lymph nodes of a representative group of OVA-sensitized and -challenged mice, treated with 0.1 g of Met-RANTES/CCL5, was analyzed by flow cytometry. The total number of cells was significantly increased after treatment with the antagonist. The proportion of cell populations in one representative experiment is shown in Fig. 7. A marked increase in the number of CD3+ T cells was observed, from $5 \pm 2 \times 10^6$ (27% CD3+/CD4+) in saline-treated mice compared with $8.3 \pm 1.4 \times 10^6$ (49% CD3+/CD4+) in the Met-RANTES/CCL5-treated mice, $p < 0.05$ (Fig. 7a). However, the proportion of activated CD4+ T cells (CD4+/CD69+) was similar in lymph nodes isolated from saline-treated (14%) and Met-RANTES/CCL5-treated (12%) mice as compared with saline-control (10%) mice, indicating that T cell activation was not impaired by treatment with the antagonist. In contrast, the proportion of B cells and APCs such as macrophages (F4/80-positive cells) and dendritic cells (CD11c-positive cells) were unchanged by Met-RANTES/CCL5 treatment (Fig. 7b).

Discussion

The treatment of inflammatory disorders by inhibiting cellular recruitment to the site of inflammation represents a novel approach because most current therapies act intracellularly on cells already recruited to the site of inflammation. However, certain anti-inflammatory therapies such as the treatment of multiple sclerosis with IFN-β, the precise mechanism of action of which is not fully delineated, is known to affect leukocyte migration (33). The chemokine system is therefore an attractive target because it is the principal orchestrator of leukocyte recruitment. Furthermore, chemokines act on 7TM receptors, which are extremely tractable targets.

Airway inflammation, particularly asthma, remains an affliction which is by no means completely therapeutically controlled. The eosinophil, along with the T lymphocyte, while widely believed to be the cell type principally responsible for the inflammation that occurs in the airways during the allergic attacks of asthma, may not necessarily be the only cell type responsible (34). Nevertheless, it is widely believed that prevention of the recruitment of eosinophils into the lung would be beneficial to asthmatic patients.

The use of N-terminally modified chemokines, including Met-RANTES/CCL5, which have greatly reduced capacities of receptor activation but retain high affinity receptor binding, in animal models of inflammation has extensively validated the therapeutic approach of blocking chemokine receptors. However, most studies...
have been conducted with single doses (15, 20, 35, 36). The chemically derived N-terminal analog, AOP-RANTES/CCL5, is a very potent inhibitor of HIV-1 infectivity in vitro (21), and moreover its potency can be attributed to its effectiveness to down-modulate CCR5 cell surface expression (23) and to prevent functional receptor to recycle (37). However, little is known about its anti-inflammatory properties. Therefore, we were interested in the comparison of the two N-terminally modified analogues Met-RANTES/CCL5 and AOP-RANTES/CCL5 in the airway inflammation model, using an extended dose response.

Both analogues demonstrated an inverse bell-shaped dose response for the inhibition of inflammatory symptoms. The inverse bell-shaped inhibition curve remains unexplained. One hypothesis is that the inhibitory effect is lost at high doses due to partial agonist activity, because both are able to induce mCCR5 down-modulation, albeit with reduced activity compared with the wild-type chemokine (38), but neither analog was able to recruit primary murine leukocytes in vitro (results not shown) or to recruit cells into the peritoneum in vivo. Moreover, the loss of inhibition due to partial agonist activity is not borne out by the observation that no increase in cellular recruitment was noted at the highest dose of 100 µg per mouse, while the full agonist RANTES/CCL5 protein was able to increase the cellularity in the BAL fluid. It is well-documented that biological effects can be lost at high doses. Chemokines typically show a bell-shaped curve in vitro chemotaxis assays, and the effect of brain-derived neurotrophic factor-mediated rescue of axotomized motor neurons decreases with increasing doses in vivo (39).

It has previously been reported that molecules which have bi-functional binding properties exert an inverse bell-shaped activity profile (25). The dose-effect relationship demonstrated by the N-terminally modified RANTES/CCL5 analogues may be complicated by the fact that chemokines bind to glycosaminoglycans on the endothelial surface and are “presented” to adherent leukocytes (40–42). Moreover, it has been shown that RANTES/CCL5, Met-RANTES/CCL5, and AOP-RANTES/CCL5 are able to aggregate on binding to immobilized heparin in vitro (43, 44). Thus, high doses of the RANTES/CCL5 analogues may result in an accumulation of the protein on the endothelial surface, thereby acting as a sink which removes the antagonist from the circulation and prevents it from binding to the receptors expressed on the surface of the circulating leukocytes. However, beyond these hypotheses, we cannot to date satisfactorily explain the mechanism underlying this observation.

The receptor binding studies revealed the surprising observation that neither the wild-type human RANTES/CCL5 nor the N-terminally modified analogues were able to compete for eotaxin/CCL11 binding to mCCR3. Yet significant reduction in eosinophil recruitment both into the BAL and to the lung tissue was observed by the administration of these analogues. It has been widely believed that CCR3 is the principal chemokine receptor responsible for the recruitment of eosinophils, and would thus be the prime target for the inhibition of their recruitment. However, our results point to the fact that eosinophil recruitment is not mediated only through this receptor, and that inhibition can be mediated through other receptors, and may possibly be attributed to upstream effects. It has been shown that Met-RANTES/CCL5 can inhibit eotaxin/CCL11-mediated eosinophil recruitment in the skin (45), but this could also be an indirect effect. It could be hypothesized that the potent effects of both analogues on inhibiting T cell chemotaxis and recruitment could result in a decreased release of eosinophil-activating factors. The hypothesis that these analogues have an effect upstream of eosinophil recruitment into the lungs is borne out by the observation that there is an accumulation of T lymphocytes in the lung-associated lymph nodes in the treated mice.

The comparison of these two analogues in this model does not show an advantage of AOP-RANTES/CCL5 over Met-RANTES/CCL5 because maximal efficacy was achieved at the 0.1 µg dose for both. The increased affinity of AOP-RANTES/CCL5 for human receptors (21, 22) is less pronounced in the murine system, where AOP-RANTES/CCL5 showed a 5-fold increase in affinity compared with Met-RANTES/CCL5 for mCCR1 and a 15-fold increase for mCCR5. This correlated with an increased potency in inhibiting the in vitro chemotaxis of murine T lymphocytes by 6- to 10-fold compared with Met-RANTES/CCL5. However, the strikingly enhanced potency demonstrated by AOP-RANTES/CCL5 in the inhibition of HIV infectivity (21) appears not to translate to such a significant improvement as a potential anti-inflammatory treatment.

The lack of effect on airway hyperresponsiveness despite the reduction in airway eosinophilia was unexpected. The relationship between eosinophil recruitment and bronchial hyperresponsiveness remains a subject of debate with evidence existing for and against the implication of this leukocyte (46, 47). The precise identification of which inflammatory chemokines are the principal mediators of airway inflammation and hyperresponsiveness is not yet fully delineated because neutralizing mAbs against MIP-1α have been reported to suppress eosinophil recruitment into the lungs, but

FIGURE 7. Flow cytometric analysis of cellular composition of lung associated lymph nodes. Mice were sacrificed 3 days after the last challenge, lung-associated lymph nodes were removed, digested by collagenase/DNase treatment and leukocytes were recovered by density gradient centrifugation. Cells were stained for flow cytometric analysis with fluorochrome-conjugated mAb specific for total leukocytes (CD45), T lymphocytes (CD3), B cells (B220), dendritic cells (CD11c), and macrophages (F4/80). Cells isolated from OVA-sensitized and -challenged mice treated with saline; cells isolated from OVA-sensitized and -challenged mice treated with 0.1 µg of Met-RANTES/CCL5 30 min before OVA-challenge. Results are representative of two independent experiments with six mice per group, and data were obtained from individual mice.
not airflow hyperresponsiveness, whereas anti-monocyte chemotactic protein-1/CCL2 mAbs failed to suppress eosinophilic inflammation, but abrogated airflow hyperresponsiveness (48). Interestingly, treatment with soluble IL-4R, as performed with these antagonists before OVA challenge in OVA-immunized mice inhibits mucus hypersecretion and eosinophil influx into the lungs, but not bronchohyperactivity to methacholine following allergen challenge (49). These findings strengthen the concept that blocking migration or activation of Th2 T lymphocytes prevents the successive events, which contribute to the pathogenesis of asthma.

The production of mucus in asthmatic patients is severely debilitating, and may even lead to death. Antichemokine receptor treatment in these experiments inhibited the production of mucus from airway epithelial cells. Although at the present we cannot rule out a direct effect of chemokines on the epithelium to induce mucus secretion, previous data using IL-5-deficient mice favor an indirect action mediated via the secretion of mediators derived from eosinophils (50) or T lymphocytes (51).

Because the chemokine system has been described by in vitro studies as being extremely redundant, one of the challenges for those in the field is to identify which receptors are playing a major role in a certain inflammatory condition. Reduction of airflow inflammation by monocyte chemotactic protein-1/CCL2 inhibition (15) highlights the importance of CCR2. Inhibition of airflow inflammation by monocyte chemotactic protein-1/CCL2 inhibits eosinophilic inflammation, but abrogates airway hyperresponsiveness (48). Inhibition of eotaxin expression (15) highlights the importance of CCR3. Inhibition of Th2 cytokine production (15) contributes to the pathogenesis of asthma.

To address the question of the importance of either CCR1 or CCR5 in airflow inflammation would be to administer these analogues to mice deficient for either receptor, but bearing in mind that compensation can always occur in knockout animals. Alternatively, the use of neutralizing mAbs which are now being developed (38), or specific receptor small molecule antagonists, provided they retain activity on the murine receptors (52), would be the ideal way to obtain a definitive answer.

Acknowledgments
We thank Frédéric Borlat and Brigitte Dufour for excellent technical assistance.

References