The Ligands of CXC Chemokine Receptor 3, I-TAC, Mig, and IP10, Are Natural Antagonists for CCR3*

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Th1 and Th2 lymphocytes express a different repertoire of chemokine receptors (CCRs). CXCR3, the receptor for I-TAC (interferon-inducible T cell α-chemoattractant), Mig (monokine induced by γ-interferon), and IP10 (interferon-inducible protein 10), is expressed preferentially on Th1 cells, whereas CCR3, the receptor for eotaxin and several other CC chemokines, is characteristic of Th2 cells. While studying responses that are mediated by these two receptors, we found that the agonists for CXCR3 act as antagonists for CCR3. I-TAC, Mig, and IP10 compete for the binding of eotaxin to CCR3-bearing cells and inhibit migration and Ca²⁺ changes induced in such cells by stimulation with eotaxin, eotaxin-2, MCP-2 (monocyte chemotractant protein-2), MCP-3, MCP-4, and RANTES (regulated on activation normal T cell expressed and secreted). A hybrid chemokine generated by substituting the first eight NH₂-terminal residues of eotaxin with those of I-TAC bound CCR3 with higher affinity than eotaxin or I-TAC (3- and 10-fold, respectively). The hybrid was 5-fold more potent than I-TAC as an inhibitor of eotaxin activity and was effective at concentrations as low as 5 nM. None of the antagonists described induced the internalization of CCR3, indicating that they lack agonistic effects and thus qualify as pure antagonists. These results suggest that chemokines that attract Th1 cells via CXCR3 can concomitantly block the migration of Th2 cells in response to CCR3 ligands, thus enhancing the polarization of T cell recruitment.

Chemokines are small secretory proteins produced by tissue cells and leukocytes that regulate leukocyte migration in inflammation and immunity (1–5). Two main subfamilies (CXC and CC chemokines) are distinguished depending on the arrangement of the first two cysteines, which are separated by one amino acid (CXC) or are adjacent (CC). All chemokines act via seven-transmembrane-domain receptors coupled to G proteins, which also form two subfamilies, CXC and CC chemokine receptors (CXCRs and CCRs). Depending on function and pathophysiological roles, it is possible to distinguish between inflammatory and homing chemokines. Inflammatory chemokines are produced in most tissues under pathological conditions upon stimulation by cytokines and bacterial toxins, whereas homing chemokines are produced constitutively at homing sites.

In the past few years it was found that lymphocytes can express most chemokine receptors in relation to their state of maturation, activation, and differentiation. CCR3, CCR5, and CXC3, for instance, are up-regulated in T cells by treatment with IL-2 and are expressed differentially in Th1 and Th2 cells (1–3). These observations explain how T cells with appropriate cytokine production and effector properties can be attracted specifically into diseased tissues. CCR3-expressing Th2 cells are recruited together with eosinophils, which express the same receptor, to sites of allergic inflammation as shown by immunopathological analysis of nasal polyps and atopic dermatitis lesions (6, 7). In such infiltrates, Th2 cells are believed to promote inflammation by releasing IL-4 and IL-5 as priming and survival factors for eosinophil and basophil leukocytes (8). CCR5, on the other hand, is characteristic for Th1 cells, which also express high levels of CXC3. Th1 cells accumulate in delayed-type hypersensitivity reactions and autoimmune inflammation (9, 10).

CCR3 binds many different CC chemokines, namely eotaxin, eotaxin-2, eotaxin-3, RANTES, MCP-2, MCP-3, and MCP-4 (4). The eotaxins are highly selective for CCR3, whereas RANTES and the MCPs recognize additional CC chemokine receptors. Eotaxin is expressed in a wide variety of cells, including eosinophils, lymphocytes, macrophages, and endothelial and epithelial cells, and is critically involved in the regulation of the basal and inflammation-dependent traffic of eosinophils (11, 12). In eotaxin-deficient mice and in animals treated with antibodies that neutralize eotaxin, eosinophil infiltration of the airways is markedly reduced (13–15). CXC3 is highly expressed on T cells activated with IL-2 and binds selectively I-TAC, Mig, and IP10 (4). Of the three ligands, I-TAC has the highest receptor affinity and is the most potent agonist, as shown by chemotaxis and Ca²⁺ mobilization assays (16). A notable feature of I-TAC, Mig, and IP10 is that their production is induced by interferon-γ, a cytokine that is typically associated with Th1 responses (16–18). IP10, for instance, is expressed in skin lesions caused by delayed-type hypersensitivity, psoriasis, and tuberculoid leprosy, where interferon-γ expression is enhanced.

CXCR chemokine receptor; I-TAC, interferon-inducible T cell α-chemoattractant; I-TAC/EoH1, I-TAC-eotaxin hybrid-1; Mig, monokine induced by γ-interferon; IP10, interferon-inducible protein 10; RANTES, regulated on activation normal T cell expressed and secreted; MCP, monocyte chemoattractant protein; IL, interleukin.

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The abbreviations used are: CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; I-TAC/EoH1, I-TAC-eotaxin hybrid-1; Mig, monokine induced by γ-interferon; IP10, interferon-inducible protein 10; RANTES, regulated on activation normal T cell expressed and secreted; MCP, monocyte chemoattractant protein; IL, interleukin.
While studying the activities of several chemokines on Th1 and Th2 cells, we observed that I-TAC, Mig, and IP10 act as antagonists for CCR3. These data suggest that CXCR3 agonists in addition to attracting CXCR3-bearing cells have the capacity to inhibit responses mediated via CCR3.

**EXPERIMENTAL PROCEDURES**

**Chemokine Synthesis**—All chemokines and chemokine analogs were synthesized chemically using tBoc (tertiary butyloxycarbonyl) solid-phase chemistry (19). They were purified by high pressure liquid chromatography and analyzed by electron spray mass spectrometry. For each chemokine used, the mass determined by mass spectrometry corresponded to the expected value.

**Cell Preparation and Culture**—Eosinophils (>95% pure) were purified from venous blood of healthy volunteers (20). A human Th2 cell line generated from cord blood cells was kindly provided by Dr. C. Chizzolini (University Hospital, Geneva, Switzerland). These cells were expanded periodically by restimulation with phytohemagglutinin in the presence of feeder cells (21).

**Chemokine Receptor Transfectants**—Murine pre-B 300-19 cells that stably express chemokine receptors were generated by transfection. cDNAs for CXCR1 (22), CXCR2 (22), CXCR4 (23), CCR1 (24) (kindly provided by Dr. P. Murphy, National Institutes of Health, Bethesda, MD), CCR3 (25), CCR5 (26), CCR5 (68), CCR5 (27), CXCR1 (28), and CXCR1 (29) were cloned into the SORuo vector (kindly provided by Dr. F. Arenzana-Seisdedos, Pasteur Institute, Paris). The cells (5 × 10⁶) were transfected by electroporation with 20 μg of linearized SORuo receptor plasmid DNA. Clones of receptor-expressing B300-19 cells were established by limiting dilution in the presence of 1.5 μg/ml puromycin (Sigma). The following receptor-transfected murine pre-B 300-19 cells were generated and characterized previously: CXCR3-B300-19 (30), CXCR5-B300-19 (31), CXCR4-B300-19 (32), CXCR7-B300-19 (33), and CXCR8-B300-19 (34). Receptor expression was determined by flow cytometry (FACSscan, Becton Dickinson, Mountain View, CA) using monoclonal antibodies to CXCR4 (12G5) (kindly provided by Dr. J. Hoxie, University of Pennsylvania, Philadelphia) and CCR3 (7B11) (35), CCR5 (5C7) (36), and CXCR3 (1C6.2) (10), all kindly provided by LeukoSite Inc. (Cambridge, MA). Labeling was done with phycocyanin-conjugated goat anti-mouse IgG (Dako). Receptor function was assessed by Ca²⁺ mobilization in response to the appropriate chemokine.

**Receptor Binding and Functional Assays**—Competition binding assays were performed with CXCR3-B300-19 cells using [³²P]-I-(4-carboxyethyl)-5-iodoacetyl-2'-deoxyuridine (I-TAC) (kindly provided by Dr. F. Arenzana-Seisdedos, Pasteur Institute, Paris). The cells (5 × 10⁶) were pretreated with I-TAC, Mig, and IP10, as shown for I-TAC in Fig. 2, in the presence or absence of I-TAC, Mig, and IP10. Shown are the average numbers of migrating cells per five high power fields in triplicate wells. The data are representative of four independent experiments.

**Inhibition of Chemotaxis**—The migration of CXCR3-B300-19 cells and human eosinophils in response to eotaxin was inhibited by I-TAC, Mig, and IP10, as shown for I-TAC in Fig. 1, A and B. The inhibition of chemotaxis induced by optimum concentrations of eotaxin was concentration-dependent and was complete at 100–1000 nM (Fig. 1, A and B). I-TAC and Mig were equally effective and somewhat more potent than IP10. None of the CXCR3 ligands induced chemotaxis of CXCR3-B300-19 cells or eosinophils (not shown).

**Inhibition of Ca²⁺ Mobilization**—As shown in Fig. 2, the [Ca²⁺]i rise induced by eotaxin in CXCR3-bearing cells was decreased in a concentration-dependent manner by pretreatment with I-TAC, which was completely inhibitory between 100 and 1000 nM. I-TAC, Mig, and IP10, on the other hand, did not induce [Ca²⁺]i changes in CXCR3-B300-19 cells or eosinophils, even at high concentrations, confirming that it is devoid of agonistic activity on CCR3 and indicating that it is a pure antagonist. Experiments with Th2 cells, which express CXCR3 and CXCR5, show that blockade of CCR3 can occur concomitantly with the activation of CXCR5 (Fig. 2B). Marked [Ca²⁺]i changes were observed after stimulation with increasing concentrations of I-TAC, which in turn prevented the response to eotaxin. Ca²⁺ mobilization induced by eotaxin in CXCR3-B300-19 cells and Th2 cells was inhibited in a concentration-dependent manner by all three CXCR3-selective chemokines (Fig. 2, C and D). I-TAC was the most potent antagonist, followed by Mig and IP10. In agreement with the chemotaxis assays, these results show that I-TAC, Mig, and IP10 significantly inhibit CXCR3-dependent responses of Th2 cells at concentrations as low as 1 nm.
CCR3-B300-19 cells were less susceptible, presumably because they express higher numbers of receptors (Fig. 2C). As several chemokines bind and activate CCR3, it was important to test the effect of the antagonists on responses induced by different ligands. As shown in Fig. 3, I-TAC prevented the \([\text{Ca}^{2+}]_i\) changes elicited in CCR3-B300-19 cells by eotaxin, eotaxin-2, MCP-2, MCP-3, MCP-4, and RANTES, demonstrating that the antagonistic effect is not dependent on the CCR3 agonist used. It was also important to test the specificity of the antagonists to block chemokine receptor-mediated responses. In a panel of 14 receptor-transfected B300-19 cell lines, I-TAC (the most potent of the three CCR3 antagonists) abrogated the \([\text{Ca}^{2+}]_i\) changes in response to stimulation with the appropriate chemokine only in CCR3-expressing cells (Fig. 4). I-TAC was slightly inhibitory on CCR5 (30–40% decrease of the \([\text{Ca}^{2+}]_i\) rise in three experiments) but had no effect on all other receptors, indicating that its antagonistic activity is restricted largely to CCR3.

**Effect of Eotaxin and MCP-4 on CXCR3-mediated Responses**—It has been reported that eotaxin and MCP-4, which are agonists for CCR3, bind to CXCR3 and that eotaxin prevents \([\text{Ca}^{2+}]_i\) changes induced by IP10 (42). In view of the observed antagonism for CCR3, we tested the effect of eotaxin and MCP-4 on CXCR3-B300-19 cells that were stimulated with I-TAC, Mig, and IP10. Neither eotaxin nor MCP-4 inhibited \([\text{Ca}^{2+}]_i\) mobilization or chemotaxis induced by I-TAC, Mig, and IP10, indicating that their effect on CXCR3 was negligible (data for I-TAC are shown in Fig. 5).

**CCR3 Antagonist Obtained by Chemokine Modification**—It has been suggested that chemokines dock onto receptors by interacting with the loop region that follows the second cysteine and that the docking facilitates a triggering of the receptor by the NH2-terminal domain (43–46). In the attempt to enhance the antagonistic effect, we synthesized a hybrid che-
mokine by substituting the NH$_2$-terminal region of eotaxin with that of I-TAC. As shown in Fig. 6, I-TAC/EoH1, corresponding to eotaxin with the first eight amino acids of I-TAC, was about 5-fold more potent than I-TAC itself as an inhibitor of CCR3-dependent [Ca$^{2+}$]$_i$ changes and chemotaxis. I-TAC/EoH1 was also tested on CXCR3-bearing cells and was found to have moderate agonistic and no antagonistic activity (data not shown).

**CCRF Binding Studies**—The relative affinities of I-TAC, I-TAC/EoH1, Mig, and IP10 to CCR3 were determined by binding competition assays with $^{125}$I-labeled eotaxin (Fig. 7). All antagonists fully displaced labeled eotaxin. I-TAC/EoH1 was the most potent competitor ($K_D$ $4.5 \pm 1.0$ nM, n = 3) followed by eotaxin ($K_D$ $13.5 \pm 1.9$ nM, n = 3) and I-TAC ($K_D$ $65.0 \pm 7.7$ nM, n = 3), whereas the affinity of Mig ($K_D$ $4065 \pm 1$, $231$ nM, n = 3) and IP10 ($K_D$ $1582 \pm 154$ nM, n = 3) was comparatively low. Overall, the binding data are in agreement with the observed antagonistic activities. The affinity of Mig, however, was lower than expected.

**CCR3 Internalization**—Binding of chemokines leads to a rapid receptor internalization, which is not observed on binding of antagonists (40, 47). Internalization was determined in cells expressing either CCR3 or CXCR3 by flow cytometry measurements of surface receptors before and after ligand exposure. As shown in Fig. 8, in CCR3-bearing cells, receptor uptake was induced by eotaxin only. In CXCR3-bearing cells, on the other hand, receptor uptake was observed with I-TAC, Mig, IP10, and I-TAC/EoH1 but not with eotaxin. Together with the functional data, these results show that the CXCR3 ligands lack agonistic activity and act on CCR3 as pure antagonists.

**DISCUSSION**

This paper shows that I-TAC, Mig, and IP10 are potent antagonists for CCR3 and prevent the responses of Th2 cells and eosinophils to CCR3-binding chemokines.

The search for chemokine antagonists began several years ago when chemokine receptor blockade was recognized as a possible therapeutic approach for inflammatory diseases. It is well established that antagonists can be generated by modifying the NH$_2$-terminal region of natural chemokines (48). Studies were performed first with IL-8 and other ELR chemokines yielding antagonists for CXCR1 and CXCR2 (49, 50). The same principle proved valid for CC chemokines, as shown by the effects obtained upon NH$_2$-terminal truncation of MCP-1, MCP-3, and RANTES (37, 51, 52). These studies indicate that, as a rule, receptor recognition depends on structural motifs located in the loop region of chemokines that follows the second cysteine. Extensive structure-activity studies of SDF-1 (stromal cell-derived factor-1) led to the proposal of a two-step interaction of chemokines with their receptors, involving specific docking via the loop region and subsequent triggering by the NH$_2$-terminal region preceding the first cysteine (43, 53).
In some cases, however, NH$_2$-terminal truncation did not yield derivatives with antagonistic properties. This result was observed with eotaxin and IP10, which lose the capacity to bind to CCR3 and CXCR3, respectively, when only a few NH$_2$-terminal residues are deleted. It was also shown that dipeptidyl-peptidase IV (CD26) reduces the activity of eotaxin by cleaving off the first two NH$_2$-terminal residues (54).

The observation that the naturally occurring I-TAC, Mig, and IP10 block CCR3 was unexpected, because normally CXC and CC chemokines discriminate precisely between CXC and CC chemokine receptors. Our data suggest that CCR3 and CXCR3, despite their overall sequence identity of only 34%, share sufficient structural similarity within domains that determine the binding of I-TAC, Mig, and IP10, on the one hand, and the binding and triggering by eotaxins, MCPs, and RANTES on the other. The existence of binding-relevant homology between CXCR3 and CCR3 is suggested in particular by the observation that the potency ranking for CCR3 antagonism, I-TAC > Mig > IP10, as shown in the present study, and the potency ranking for agonistic activity via CXCR3 as determined by Cole et al. (16) are the same. It is also noteworthy that NH$_2$-terminal truncation of IP10 leads to a loss of agonistic activity on CXCR3 as well as a loss of antagonistic effects on CCR3 (data not shown). We found that CXCR3 ligands block CCR3, but we were unable to demonstrate the converse; this contrasts with the report of Weng et al. (42) and suggests that CCR3 ligands are unlikely to exert biologically relevant effects via CXCR3. It has been reported that murine secondary lymphoid tissue chemokine has agonistic activity on murine CXCR3 (55). Human secondary lymphoid tissue chemokine, on the other hand, is inactive on human and murine CXCR3 (Ref. 56; data not shown).

Attempts to design high affinity antagonists by NH$_2$-terminal truncation of eotaxin have been unsuccessful. This paper presents an alternative approach. After realizing that CXCR3 and CCR3 receptors may have homologous binding domains, we synthesized a hybrid by replacing the NH$_2$-terminal region of eotaxin with that of I-TAC. Eotaxin was chosen because it has the highest affinity for CCR3 and I-TAC because it is the best CCR3 antagonist. Substitution of the NH$_2$-terminal region led to the loss of receptor triggering activity, whereas retaining high affinity binding yielded a chemokine analog with higher affinity to CCR3 than eotaxin and I-TAC.

Several lines of evidence indicate that I-TAC, Mig, IP10, and I-TAC/EoH1 are pure CCR3 antagonists. These ligands did not induce chemotaxis or [Ca$^{2+}$], changes in CCR3-bearing cells and did not induce CCR3 internalization, which is an agonist-mediated event due to the phosphorylation of the receptor by G protein-coupled receptor kinases and subsequent uptake in clathrin-coated pits (57). Several chemokine receptors, including CXCR1 and CXCR2 (59), CXCR4 (40, 60, 61), CCR1 (62), CCR2 (63), and CCR5 (40, 41, 47, 64), are known to be internalized after agonist binding. Different chemokines that bind to the same receptor can induce differential internalization as recently reported for CCR3 (65, 66) and CCR5 (47). In agreement with these findings, CXCR3 was internalized to different extents by I-TAC, Mig, IP10, and I-TAC/EoH1.

Inflammatory and immune reactions are characterized by the production of chemokines in the affected tissues, leading to the infiltration of leukocytes that bear the appropriate receptors. The local expression of chemokines is often induced by cytokines. In the context of this paper, it is important to realize that I-TAC, Mig, and IP10, which attract CXCR3-bearing cells, are induced by interferon-γ (16–18), whereas eotaxin, a specific agonist for CCR3, is induced by IL-4 (67). The infiltrate observed in the presence of interferon-γ is rich in Th1 cells, whereas Th2 cells predominate under the influence of IL-4. The present observations suggest that CXCR3-selective chemokines enhance this polarization by acting as antagonists of CCR3 and thus inhibiting the infiltration of Th2 cells, in addition to their effect as attractants of Th1 cells via CXCR3. This paper describes a new mechanism for the regulation of leukocyte recruitment by chemokines based on the combination of agonistic and antagonistic effects.

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