Brief Definitive Report

Eotaxin-2, a Novel CC Chemokine that Is Selective for the Chemokine Receptor CCR3, and Acts Like Eotaxin on Human Eosinophil and Basophil Leukocytes

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Summary

A novel human CC chemokine consisting of 78 amino acids and having a molecular mass of 8,778.3 daltons (VVPSPCCMF FVSKRPENRKVSQSSRS TCLKAVIFT TKKGQPSGDPKQEVKQRMKNLDAKQKKA SPRARAVA) was isolated together with three minor COOH-terminally truncated variants with 73, 75, and 76 residues. The new chemokine was termed eotaxin-2 because it is functionally very similar to eotaxin. In terms of structure, however, eotaxin and eotaxin-2 are rather distant, they share only 39% identical amino acids and differ almost completely in the NH₂-terminal region. Eotaxin-2 induced chemotaxis of eosinophils as well as basophils, with a typically bimodal concentration dependence, and the release of histamine and leukotriene C₄ from basophils that had been primed with IL-3. In all assays, eotaxin-2 had the same efficacy as eotaxin, but was somewhat less potent. The migration and the release responses were abrogated in the presence of a monoclonal antibody that selectively blocks the eotaxin receptor, CCR3, indicating that eotaxin-2, like eotaxin, acts exclusively via CCR3. Receptor usage was also studied in desensitization experiments by measuring [Ca²⁺]i changes in eosinophils. Complete cross-desensitization was observed between eotaxin-2, eotaxin and MCP-4 confirming activation via CCR3. No Ca²⁺ mobilization was obtained in neutrophils, monocytes and lymphocytes, in agreement with the lack of chemotactic responsiveness. Intradermal injection of eotaxin-2 in a rhesus monkey (100 or 1,000 pmol per site) induced a marked local infiltration of eosinophils, which was most pronounced in the vicinity of postcapillary venules and was comparable to the effect of eotaxin.

Leukocyte recruitment in inflammation and immunity is regulated by a large number of CXC and CC chemokines (1). In the past few years several new proteins of this class have been discovered and considerable information has been gained about the functions of the known ones. It has been shown in particular that the monocyte chemotactic proteins (MCP-1, MCP-2, MCP-3, and MCP-4) do not only act on monocytes, as their name suggests, but also on lymphocytes (2, 3) and basophils (4, 5), and that MCP-2, MCP-3 and MCP-4 are potent attractants for eosinophils (6–8).

Eosinophilia and tissue infiltration by eosinophils are frequently observed in allergic inflammation and parasitic diseases (9). The mechanisms by which these cells are recruited and activated is widely studied because of the pathological consequences resulting from the release of their phlogogenic and cytotoxic products. The challenge is to understand the selectivity of the chemotactic process and to develop efficient inhibitors. Several factors have been proposed as eosinophil attractants such as the anaphylatoxin C5a (10), platelet-activating factor (11), and more recently RANTES (12) and IL-16 (13). None of these stimuli is selective, however, and the discovery of eotaxin, which was originally shown to attract only eosinophils and to be specific for a single chemokine receptor, CCR3 (14–16), was greeted as a promising advance (17).

Within a large-scale sequencing and expression program for the discovery of new chemokines, we have recently identified MCP-4, a CC chemokine with powerful effects on eosinophils (8). In this paper, we describe a chemokine that is also active on eosinophils and is functionally very similar to eotaxin. It attracts and activate human eosino-
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Eotaxin-2 Structure. The purified material contained a protein of ~8.5 kD, as judged on SDS–PAGE. The material was treated with GluC under conditions ensuring cleavage COOH-terminally of glutamic acid, and three fragments were obtained. The first two were homogeneous and extended from residue 1 to 18 and 19 to 54, respectively, while the third showed some COOH-terminal heterogeneity. Sequencing and mass spectrometry revealed amino acid sequences of eotaxin-2 aligned with eotaxin, MCP-3, MCP-4, MIP-1α, and RANTES. Identical amino acids are indicated by a hyphen. The sites of COOH-terminal truncation leading to the shorter variants are indicated by arrowheads.

Figure 1. Amino acid sequences of eotaxin-2 aligned with eotaxin, MCP-3, MCP-4, MIP-1α, and RANTES. Identical amino acids are indicated by a hyphen. The sites of COOH-terminal truncation leading to the shorter variants are indicated by arrowheads.

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Materials and Methods

Cloning, Expression, Purification and Analysis. The EST representing CKβ-6 cDNA was identified in the database of Human Genome Sciences Inc. (Rockville, MD) on the basis of the CC motif and the homology to known CC chemokines (18). The cDNA was isolated from a library derived from activated human monocytes, and the mature protein was expressed in Sf9 insect cells (CRL 1711; American Type Culture Collection, Rockville, MD). Purification was performed by cation exchange, heparin affinity, and size exclusion chromatography (poros 50 HS, poros 20 HE1; Perseptive Biosystem; and Sephacryl S200 HR; Pharmacia) in the presence of protease inhibitors (20 mg/ml Pefabloc SC; Boehringer Mannheim, 1 mg/ml leupeptin, 1 mg/ml E64, and 1 mM EDTA). The purified protein was analyzed by laser desorption mass spectrometry (matrix-assisted laser desorption ionization-time of flight) and by Edman degradation after partial proteolysis with the endoprotease GluC (Boehringer Mannheim).

Chemokines. Eotaxin, MCP-3, MCP-4, RANTES, and MIP-1α were used as standards. MCP-4 was cloned and expressed as described previously (8), and the other chemokines were chemically synthesized by Dr. I. Clark-Lewis (Biomedical Research Centre, University of British Columbia, Vancouver, Canada) (19).

CCR3-blocking Antibody. The anti-CCR3 monoclonal antibody 7B11 which selectively blocks the eotaxin receptor (20) was kindly provided by Dr. Charles Mackay (LeukoSite Inc., Cambridge, MA).

Cells. Monocytes (21), lymphocytes (3), and neutrophils (22) were isolated from donor blood buffy coats. The lymphocytes (12) and basophils (23, 24) were purified from the venous blood of healthy volunteers. The eosinophil preparations were 90% pure and the basophil preparations consisted of 70–80% basophils and 20–30% lymphocytes.

[Ca2+]i Changes. Changes in the cytosolic free Ca2+ concentration ([Ca2+]i) were assayed in monocytes, eosinophils, lymphocytes, and neutrophils loaded with Fura-2 (25). Receptor desensitization was tested by monitoring [Ca2+]i changes in response to sequential stimulation with chemokines (21).

In Vitro Chemotaxis. Chemotaxis was assessed in 48-well chambers (Neuro Probe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes (Nucleopore, Neuro Probe, Cabin John, MD) with 5-μm pores for eosinophils, basophils, neutrophils and monocytes, and 3-μm pores for lymphocytes (3, 6). Cell suspensions and chemokine dilutions were made in RPMI 1640 supplemented with 20 mM Hepes, pH 7.4, and 1% pasteurized plasma protein solution (Central Laboratory of the Swiss Red Cross). Migration was allowed to proceed for 60 min at 37°C in 5% CO2. The membrane was then removed, washed on the upper side with PBS, fixed, and stained. All assays were done in triplicate, and the migrated cells were counted in five randomly selected fields at 1,000-fold magnification. Spontaneous migration was determined in the absence of chemoattractant.

Histamine and Leukotriene C4 (LTC4) Release. Basophils (0.1–0.3 × 106 cells/ml) in 20 mM Hepes, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 0.5 mM glucose and 0.025% BSA were warmed to 37°C, primed with 10 ng/ml IL-3 for 5 min, and then challenged with a chemokine. The reaction was stopped on ice after 20 min and histamine and LTC4 were measured in the supernatants (26). Histamine release was expressed as percent of the total cellular content (determined after cell lysis). LTC4 generation was expressed in ng per 105 basophils.

Enzyme Release. N-acetyl-β-D-glucosaminidase release was assayed in monocytes (21) and elastase release in neutrophils (22) exactly as described previously.

In Vivo Activity. A male thseus monkey of 7.5 kg was anesthetized by injection of 10 mg/kg Ketamine (Ketalar, Parke Davis) i.m. and 15 mg/kg Na Thiopental (Pentotal, Abbott) i.v. The chemokines (100 pmol eotaxin, 100 and 1,000 pmol eotaxin-2 in 100 μl pyrogen-free isotonic saline) were then administered intradermally on the back, and full skin thickness punch biopsies of 8-mm diameter were taken from the injection sites after 4 h. The biopsies were fixed in formalin, embedded in paraffin, and 5-μm sections were prepared. The sections were stained with Giemsa solution or hematoxylin and eosin, and the eosinophil infiltrates were evaluated by two independent observers. In each section, eosinophils were counted at a magnification of 630× in five randomly selected fields including a postcapillary venule, using a grid of 0.19 × 0.19 mm, and the number of eosinophils per mm2 was calculated. Photographs were taken at a magnification of 1,000× with a Nikon Microphot microscope (Nikon AG, Switzerland).

Results and Discussion

Eotaxin-2 Structure. The purified material contained a protein of ~8.5 kD, as judged on SDS–PAGE. The material was treated with GluC under conditions ensuring cleavage COOH-terminally of glutamic acid, and three fragments were obtained. The first two were homogeneous and extended from residue 1 to 18 and 19 to 54, respectively, while the third showed some COOH-terminal heterogeneity. Sequencing and mass spectrometry revealed amino acid sequences of eotaxin-2 aligned with eotaxin, MCP-3, MCP-4, MIP-1α, and RANTES. Identical amino acids are indicated by a hyphen. The sites of COOH-terminal truncation leading to the shorter variants are indicated by arrowheads.

Eotaxin-2, a Functional Analogue of Eotaxin

Philips and basophils via the eotaxin receptor, CCR3, and has no activity on other leukocytes. Because of these properties, we have named the novel chemokine eotaxin-2.
tree COOH-terminally truncated forms consisting of residues 55–73, 55–75, and 55–76 in addition to the main form extending from residue 55 to 78 (Fig. 1). The mass of the 78-residue form of eotaxin-2 is 8,778.3 and the amino acid identities to reference chemokines were 43% for MCP-4, 42% for MIP-1α/H9251, 39% for MCP-3 and eotaxin, and 32% for RANTES. Since the four COOH-terminal variants could not be separated all tests were performed with the mixture. The sequence shown in Fig. 1 corresponds to the cDNA-deduced sequence of the chemokine MPIF-2 that was shown to inhibit the proliferation of myeloid progenitors (18), except for two substitutions, Ala35 for Gly and Ser47 for Phe.

Figure 2. Cross-desensitization of human blood eosinophils. Fura-2–loaded cells were stimulated sequentially at 90-s intervals with 50 nM eotaxin-2 and another CC chemokine at the same concentration, and [Ca²⁺]i dependent fluorescence changes were recorded. The tracings are representative for three separate experiments performed under identical conditions with cells from different donors.

Leukocyte Responses and Receptor Usage. [Ca²⁺]i changes, were monitored in blood leukocytes after stimulation with 1 to 1,000 nM eotaxin-2. A marked, concentration-dependent effect was observed on eosinophils whereas neutrophils, monocytes and T lymphocytes did not respond. Desensitization experiments were then performed with eosinophils to gain information on receptor selectivity. As shown in Fig. 2, eosinophil stimulation with eotaxin-2 abrogated the response to eotaxin, attenuated the responses to MCP-3 and RANTES, but did not appreciably affect the response to MIP-1α. In agreement with these results the [Ca²⁺]i rise induced by eotaxin-2 was abrogated by prior stimulation with eotaxin, decreased by stimulation with RANTES or MCP-3, but was not affected by stimulation with MIP-1α. Cross-desensitization was also observed between eotaxin-2 and MCP-4 (data not shown). These results suggest that eotaxin-2 is a selective agonist for eosinophils. The complete cross-desensitization between eotaxin-2 and eotaxin indicates that both chemokines act mainly, if not exclusively, via CCR3. This conclusion is in agreement with the partial desensitization of the responses to RANTES and MCP-3, which are known to interact with at least two additional receptors, CCR1 and CCR2. The lack desensitization of the response to MIP-1α, on the other hand rules out an interaction of eotaxin-2 with CCR1.

In Vitro Chemotaxis. As shown in Fig. 3, eotaxin-2 was a highly effective attractant for eosinophils and basophils. The concentration dependence was typically bimodal, and the overall effect was similar to that of eotaxin. Maximum migration was reached at 100 nM eotaxin-2 and 10 nM eotaxin which thus appears to be more potent. When the assay was performed in the presence of the anti-CCR3 antibody, eosinophil and basophil chemotaxis toward either eotaxin or eotaxin-2 was completely prevented, indicating that they both acted exclusively via CCR3. Eotaxin-2, like eotaxin, did not induce migration of neutrophils, monocytes and T lymphocytes, which is in agreement with the lack of Ca²⁺ mobilization observed in these cells.

Histamine and LTC4 Release. In view of the marked chemotactic activity toward basophils, the effect of eotaxin and eotaxin-2 as inducers of release was determined (Fig. 4). Both chemokines induced a similar release of histamine.
and peptido leukotrienes in IL-3 pretreated basophils from several unselected donors. The curves relating effect to concentration show that eotaxin was somewhat more potent than eotaxin-2 in particular as inducer of LTC4 release. Similar release responses were obtained with RANTES and MIP-1α (data not shown). In agreement with the data of the chemotaxis assays, mediator release induced by eotaxin and eotaxin-2 was markedly inhibited by pretreatment of the cells with the anti-CCR3 antibody.

**Effects In Vivo.** The ability of eotaxin-2 to act as chemoattractant in vivo was examined in a rhesus monkey in comparison with eotaxin. As shown in Fig. 5A, 4 h after injection of 100 pmol per site both chemokines induced a similar, marked eosinophil infiltration whereas the vehicle alone had no effect. The number of infiltrating eosinophils was ≈50% higher at sites where 1,000 pmol eotaxin-2 was applied. The effect is remarkable because the monkey used in this experiment had a low blood eosinophil count (0.7% of total leukocytes). As shown by representative micrographs, several infiltrating eosinophils can be recognized by the characteristic nucleus and the Giemsa staining of the granules. They are found in the vicinity of postcapillary venules (Fig. 5B and C) and in association with the venular wall (Fig. 5C).

This paper describes a chemokine which we have termed eotaxin-2 because its function is perfectly analogous to that of eotaxin. Both chemokines activate and attract eosinophils and basophils, but no other leukocytes, and appear to act exclusively via CCR3. It must be mentioned, however, that a virtually identical chemokine, MPIF-2, was reported to inhibit colony formation by myeloid progenitor cells (18). The functional similarity between eotaxin and eotaxin-2 is astonishing because these chemokines share only 39% identical amino acids. In addition, they differ almost completely within the short NH₂-terminal sequence preceding the first cysteine, which has been identified as the receptor triggering region of several chemokines.

![Figure 4](image1.png)

**Figure 4.** (A and B) Histamine and LTC₄ release by basophils pretreated with IL-3 in response to increasing concentrations of eotaxin and eotaxin-2. Each symbol represents mean values from four experiments performed with cells from different donors. (C and D) Histamine and LTC₄ release by IL-3 pretreated basophils after stimulation with 100 nM eotaxin or eotaxin-2 in the presence (open columns) or absence (filled columns) of anti-CCR3. Mean ± SEM of three experiments performed with basophils from different donors.

![Figure 5](image2.png)

**Figure 5.** (A) In vivo infiltration of eosinophils. A rhesus monkey received intradermal injections of eotaxin-2 (100 or 1,000 pmol per site), eotaxin (100 pmol per site) or pyrogen-free isotonic saline. After 4 h, punch biopsies were taken and processed for histology. Eosinophils were counted on sections stained with hematoxylin and eosin (five fields per section) in areas including postcapillary venules. (B) Micrograph of a skin site 4 h after injection of 100 pmol eotaxin-2, stained with Giemsa solution. Clearly identifiable eosinophils are seen near the inset; one is seen in contact with the venule wall. (C) Micrograph of a skin site 4 h after injection of 1,000 pmol eotaxin-2, stained with haematoxylin and eosin. Two eosinophils are within the venule wall (see also inset) and others are in the lumen and in the tissue.
It is well established that all CXC chemokines that activate neutrophils via the IL-8 receptors, CXCR1 and CXCR2, share a conserved Glu-Leu-Arg motif preceding the first cysteine that tolerates only minimal substitutions (28, 29), and it has been shown more recently that CC chemokines drastically lose activity toward monocytes (1) and basophils (27, 30) upon deletion of as few as one or two NH$_2$-terminal amino acids. Yet the present findings show selective activation of the same receptor, CCR3, by two chemokines that have only one common residue (Pro-4 of eotaxin-2 corresponding to Pro-6 of eotaxin) in the presumed receptor triggering domain. The eotaxin sequence is actually much more similar (~60% identity) to that of MCP-3, which binds to CCR3, but also to CCR1 and CCR2 and even to MCP-1, a selective ligand for CCR2, which is inactive on eosinophils.

Eotaxin is an unusually selective chemokine (17). It was discovered as attractant for eosinophils in the bronchoalveolar lavage fluid obtained in an experimental model for lung allergy in guinea-pigs (17, 31) and subsequently shown to occur in mice (32) and humans (15) as well. The present discovery of a chemokine that shares with eotaxin receptor specificity, in vitro activities on eosinophils and basophils, and the ability to elicit eosinophil infiltration in vivo reveals the existence of a mechanism for the concomitant and selective recruitment of the two types of granulocytes that are characteristic of allergic inflammation.

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