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Cloning and Characterization of the Guinea Pig Eosinophil Eotaxin Receptor, C-C Chemokine Receptor-3: Blockade Using a Monoclonal Antibody In Vivo

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Certain C-C chemokines, signaling via the eotaxin receptor C-C chemokine receptor-3 (CCR3), are thought to be central mediators of eosinophil accumulation in allergic inflammation. To investigate the role of CCR3 in vivo, we cloned the guinea pig eotaxin receptor (guinea pig CCR3) from a genomic DNA library. We isolated a single-exon open reading frame coding for a 358-amino acid chemokine receptor protein with 67 and 69% homology to human and murine CCR3, respectively. When expressed in stable transfecants, this receptor bound

125I-labeled guinea pig eotaxin, 125I-labeled human monocyte chemotactic protein-3, and 125I-labeled human RANTES. In chemotaxis assays, guinea pig CCR3 transfecants responded only to guinea pig eotaxin, with a maximal effect at 100 nM. mAbs were raised that bound selectively to both guinea pig CCR3 transfecants and guinea pig eosinophils. One of these mAbs, 2A8, blocked both ligand binding to transfecants and their chemotaxis in response to eotaxin. The Ab also inhibited chemotaxis and the elevation of cytosolic calcium in guinea pig eosinophils in response to eotaxin. F(ab′)2 fragments of 2A8 were prepared that retained the ability to inhibit eosinophil calcium responses to eotaxin. Pretreatment of

111In-labeled eosinophils in vitro with F(ab′)2 2A8 selectively inhibited their accumulation in response to eotaxin in vivo. These data demonstrate that functional blockade of eosinophil chemokine receptors can be achieved in vivo and provide further support for the development of novel anti-inflammatory drugs targeting eosinophil recruitment through chemokine receptor antagonism. The Journal of Immunology, 1998, 161: 6139–6147.

The acute asthmatic attack is associated with a marked recruitment of eosinophils into the lung. The subsequent activation of these eosinophils is thought to be a major contributor to the observed histopathologic features of lung damage such as epithelial shedding and to the underlying bronchial hyperreactility that is a hallmark of asthma (1–4).

Eosinophils accumulate from the microcirculation in response to locally generated chemoattractants. Several types of chemoattractants are active on eosinophils in vitro (4, 5), but in allergic inflammation in vivo a central role is emerging for the C-C class of chemokines, in particular eotaxin (6–12). Eotaxin was first identified as the major eosinophil chemoattractant present in bronchoalveolar lavage fluid from a guinea pig model of allergic airway inflammation (6, 13), and using primers based initially on the guinea pig protein sequence, guinea pig (14, 15), murine (16), rat (17), and human eotaxin (8) have been cloned. In sensitized guinea pigs, the elevation in eotaxin concentrations in the lung tissue of allergen-challenged animals parallels the onset of eosinophil accumulation, and all the eosinophil chemoattractant activity in bronchoalveolar lavage fluid obtained 6 h after pulmonary allergen challenge is neutralized by an anti-eotaxin Ab (10).

Further studies have investigated in more detail the mechanisms of eosinophil accumulation in vivo, central to which is the generation of a blood eosinophilia (7). We have previously obtained evidence that effective eosinophil accumulation in response to local eotaxin generation in tissues is dependent upon the elevation of blood eosinophil levels, mediated by the actions of IL-5 to release a rapidly mobilizable bone marrow pool of eosinophils (7, 10). Recently, we have shown that eotaxin alone or acting in synergy with IL-5 can mobilize bone marrow eosinophils and their progenitors (18), supporting an important role for eotaxin in eosinophil recruitment acting through both local and remote pathways. Evidence is also accumulating for an important role for eotaxin in human disease. Eotaxin mRNA and protein are up-regulated in atopic asthma, and eotaxin contributes significantly to the eosinophil chemotactic activity present in bronchoalveolar lavage fluid from asthmatic patients (19–21). Eotaxin has also been implicated in eosinophil accumulation in other human diseases (22).

Chemoattractants, including chemokines, signal through seven-transmembrane, G protein-coupled receptors (23–26). Guinea pig eotaxin was originally shown to be a potent stimulator of both guinea pig and human eosinophils (6). Binding and desensitization studies with eotaxin and human RANTES (hRANTES)3 on human eosinophils predicted a common receptor for these chemokines (8). The human eosinophil receptor, C-C chemokine receptor-3 (CCR3),

3 Abbreviations used in this paper: h, human; CCR3, C-C chemokine receptor-3; MCP, monocyte chemotactic protein; rMC5a, recombinant human C5a; PMNL, polymorphonuclear leukocyte; LTB4, leukotriene B4.
was subsequently cloned from eosinophil cDNA using primers and probes based upon the human CCR1 and other chemokine receptor sequences (27, 28). Murine CCR3 has been cloned and characterized using similar strategies (29, 30).

Studies using an anti-human CCR3 mAb have confirmed CCR3 to be the major eosinophil chemokine receptor in the majority of the human population (12, 27), although in some individuals, eosinophils also show potent responses to chemokines including hRANTES and MIP-1α via another receptor (Ref. 31 and I. Sabroe et al., unpublished data). Furthermore, eotaxin and its recently described functional homologue eotaxin-2 (32, 33) are unusual among C-C chemokines in that they show marked receptor selectivity, signaling only via CCR3 (8, 28, 32, 33). In humans, CCR3 is also expressed on basophils (34) and some Th2 cells (35–38), supporting an important role for CCR3 signaling pathways in allergic inflammation.

Blockade of CCR3 may therefore provide an effective therapy in the treatment of allergic diseases including asthma, and the development of CCR3 antagonists has been identified as a major therapeutic target (39). To understand better the role of eotaxin and its receptor in allergic inflammation, we have cloned and characterized the guinea pig eosinophil receptor, guinea pig CCR3. We have produced a blocking mAb to this receptor and demonstrate its ability to block the actions of eotaxin on eosinophils in vivo.

Materials and Methods

Materials

The Dunkin-Hartley guinea pig liver genomic DNA library in EMBL3A was purchased from Stratagene Cloning Systems (La Jolla, CA). 125I-labeled human monocyte chemotactic protein (hMCP)-3 and 125I-labeled hRANTES (each 2200 Ci/mmol) were from New England Nuclear (Boston, MA). Cell culture media and reagents including RPMI 1640, HEPS, FCS, sodium bicarbonate, sodium pyruvate, 2-ME, and genetin (G418) were obtained from Life Technologies (Paisley, U.K.). Percoll, BSA, and general laboratory reagents were obtained from Sigma (Poole, U.K.). Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Human IL-8 was produced in Escherichia coli as described (40). Human eotaxin was purified from SF-1 insect cell culture supernatants infected with a recombinant baculovirus baculovirus obtained using the Bac-to-Bac baculovirus expression system (Life Technologies). All other chemokines other than those below were from Peprotech EC, (London, U.K.). Recombinant human C5a (rhC5a) was from Dr. J. van Oostrum (Ciba-Geigy, Summit, NJ). ENA-2 anti-human E-selectin F(ab')2 fragments were from Dr. B. Guarino, G. Andrews, and H. Showell (Pfizer, Groton, CT). Recombinant human (27) and mouse (29) CCR3 cDNA sequences as probes. Plaques showing hybridization to both probes under high stringency wash conditions were cloned by limiting dilution and rescreened for their ability to bind 125I-labeled hMCP-3 (described below) and their ability to migrate to guinea pig eotaxin.

Purification of guinea pig leukocytes

Guinea pigs (400–450 g) were humanly killed by the administration of an overdose of sodium pentobarbital i.p. (May and Baker, Oxford, U.K.), and blood was taken by cardiac puncture into 10 mM EDTA. For some experiments, guinea pigs were pretreated with 1 μg of an antihuman IL-5 i.v. 90 min before euthanasia. The whole blood was spun at 260 × g for 20 min and the plasma discarded. Dextran (5 mL; 9% in normal saline) and 20 mL of normal saline were added per 15 mL of the Buffy coat/erythrocyte cell pellet and gently mixed, and the erythrocytes were allowed to sediment over 30 min. The leukocyte-rich suspension was harvested, and the leukocytes were pelleted by centrifugation. The mononuclear cells and polymorphonuclear leukocytes (PMNLs, comprising eosinophils and neutrophils) were separated over a two-layer 70%/80% discontinuous PBS/Percoll gradient centrifuged at 400 × g for 25 min. Contaminating erythrocytes were removed by hypotonic shock lysis (41). The cells were washed in the appropriate assay buffer and counted using Kimura's stain.

Chemotaxis assays

Chemotaxis experiments with L1.2 cells or L1.2 receptor transfected cell lines were performed using Transwell tissue culture inserts (Costar) as described (8).

For guinea pig leukocyte chemotaxis, PMNLs were prepared from IL-5-treated animals as above, and the chemotaxis assay was performed as described (18). Briefly, aliquots of 5 × 10^6 PMNLs (containing 18% ± 3.2% (SD) eosinophils) were pretreated for 100 μL of buffer (RPMI 1640 containing 20 mM HEPS and 0.25% BSA) with or without 0.25 μM guinea pig eotaxin for 10 min at room temperature. The cells were transferred to Transwell inserts within 24-well tissue culture plates containing 400 μL of buffer, guinea pig eotaxin, or rhC5a per well. The plates were incubated for 1 h at 37°C, and the chemotactic responses were determined using FACS analysis to measure the eosinophil recruitment to the lower chamber, as described previously (18).

Radiolabeled ligand binding assays

Chemokine binding studies of transfected cells using 0.1 nM 125I-labeled hMCP-3 or 0.1 nM 125I-labeled hRANTES as the ligand were performed using a modified method previously reported (8). Briefly, cells were washed once in PBS and resuspended in buffer (1 × 10^5 cells/ml in PBS with 50 mM HEPS, pH 7.5; 1 mM CaCl2, 5 mM MgCl2, 0.5% BSA; and 0.05% sodium azide). Aliquots of 50 μL of 10^5 cells) were dispensed into 1.5-ml Eppendorf tubes, followed by the addition of cold competitor and radiolabeled chemokines as indicated. The final reaction volume was 200 μl. Nonspecific binding was determined in the presence of 500 nM unlabeled MCP-3. After a 60-min incubation at 37°C, the cells were washed twice with 200 μl of buffer containing 0.5 M NaCl. Cell pellets were then counted. The data are presented as the percentage of specific binding calculated according to the formula 100 × (S - B)/(T - B), where S is the radioactivity of the sample, B is background nonspecific binding, and T is total binding without competitors. Duplicates were used throughout the experiments, and the SDs were always less than 10% of the mean. All experiments were repeated at least three times. Curve fit was calculated by Kaleidagraph software (Synergy Software, Reading, PA). In some experiments, binding of 125I-labeled hMCP-3 and 125I-labeled hRANTES was performed in the RPMI-based buffer as used for chemotaxis assays (see above).

Binding assays using 125I-labeled guinea pig eotaxin were performed using a modified previously described method (41). Briefly, guinea pig eotaxin was iodinated using the iodogen (Pierce & Warriner, Chester, U.K.) method as described (42), with a specific activity of 195 Ci/mmol. Transfectants were incubated with 0.3 nM 125I-labeled guinea pig eotaxin in RPMI 1640 + 20 mM HEPS + 0.5% BSA + 0.05% sodium azide (pH 7.4) and varying concentrations of unlabeled chemokines (in a final volume of 50 μL) at room temperature for 60 min. Fifty microliters of assay buffer containing NaCl was added to a final concentration of 0.5 M NaCl, and the samples were mixed and layered onto tubes containing 150 μL of silicone oil. The cells were pelleted through the oil by centrifugation (13,000 × g for 5 min at 15°C). The cell pellet and supernatant were counted separately.
in a Canberra Packard Cobra 5010 gamma counter (Canberra Packard, Pangebourne, U.K.). Data are presented without the subtraction of nonspecific binding. Curve fits and calculation of ligand \( K_d \) were performed using MacLigand software (NIH version 4.92).

**Measurement of cytosolic calcium in purified guinea pig eosinophils**

Guinea pig peritoneal eosinophils were elicted in response to horse serum and purified over discontinuous Percoll gradients as previously described (5). Eosinophils (1 \( \times 10^7/ml \)) were loaded with 1 \( \mu M \) fura 2 as previously described (41), washed in PBS + 0.1% BSA, and resuspended at a final concentration of 1 \( \times 10^6 \) cells/ml in PBS + 0.25% BSA + 10 mM HEPES + 10 mM glucose. Aliquots of cells (2 ml) were dispensed into quartz cuvettes and equilibrated with 1 mM calcium at 37°C for 10 min before use. Changes in fluorescence were measured with a Perkin-Elmer (Norwalk, CT) LS-50 fluorescence spectrophotometer as previously described (41).

Calculation of intracellular free calcium was derived from the fluorescence spectra (excitation wavelengths, 340 and 380 nm; emission wavelength, 510 nm) in accordance with established methods (41).

**Generation of anti-guinea pig CCR3 receptor mAbs and flow cytometry**

mAbs reactive with guinea pig CCR3 were generated by immunizing C57BL/6 mice with 1–2 \( \times 10^7 \) receptor transfecteds i.p. six to eight times at 2-wk intervals. The final immunization was given i.v. Three days later, the spleen was removed and the splenic lymphocytes were fused with the SP2/0 cell line as described (27). Candidate anti-guinea pig CCR3 mAbs were initially identified by flow cytometry. Hybridoma supernatants were incubated with guinea pig CCR3 receptor transfecteds and untransfected L1.2 cells, and bound Ab were detected using FITC-conjugated F(ab\(^\prime\))\(_2\) goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). mAbs showing selectivity for guinea pig CCR3 transfecteds were cloned by limiting dilution and rescreened against a panel of L1.2 cell chemokine receptor transfecteds and guinea pig leukocytes. For flow cytometry of guinea pig leukocytes, 5 \( \times 10^6 \) cells were incubated with mAbs in 100 \( \mu l \) of buffer (PBS containing 10 mM HEPES, 10 mM glucose, and 0.1% BSA) for 60 min on ice, washed twice (220 \( \times g \) for 7 min), and incubated for 30 min at 4°C with FITC-conjugated F(ab\(^\prime\))\(_2\) goat anti-mouse IgG. The cells were washed once and resuspended in PBS before FACS analysis.

**Generation of F(ab\(^\prime\))\(_2\) fragments of mAb 2A8**

For use in vivo, F(ab\(^\prime\))\(_2\) fragments of mAb 2A8 were generated by TSD BioServices (Germantown, NY) as follows. The 2A8 (IgG2a) Ab was first purified from cell culture supernatant by passage over a protein A-Sepha-

**Inhibition of \(^{111}\)In-labeled eosinophil accumulation by mAb pretreatment in vivo**

Eosinophils from four donor animals were purified over discontinuous Percoll gradients, pooled, and labeled with \(^{111}\)InCl\(_3\), as previously described (5). The radiolabeled eosinophils were divided into two aliquots and pre-

**FIGURE 1.** Sequence of the guinea pig eotaxin receptor, guinea pig CCR3. A 1.1-kb sequence was isolated from a guinea pig genomic DNA library encoding a 358-amino acid protein with 67 and 69% homology to human and murine CCR3, respectively. The predicted protein sequence of the open reading frame is shown above, together with the sequences of human and murine CCR3, with human CCR1 and CCR2b for comparison. Residues showing homology with these comparative sequences are shown in boldface type. The sequence has been deposited in the GenBank database and assigned accession number AF060698.
FIGURE 2. a, The ligand binding of the guinea pig CCR3 stable transfectant cell line 4B4. i, Transfectants were incubated with 0.3 nM 125I-labeled guinea pig eotaxin and varying concentrations of unlabeled guinea pig eotaxin. After washing, the transfectant cell pellets were counted in a gamma counter, and the results are presented as a percentage of maximum binding. All experiments were performed in triplicate, and data shown are the means ± SEM of three or four experiments. ** signifies p < 0.01, and *** signifies p < 0.001 (both significant competitions; ANOVA and Bonferroni's post test). ii, Competition of 0.1 nM 125I-labeled hMCP-3 binding is displayed as a percentage of maximum specific binding. iii, Competition of 0.1 nM 125I-labeled hRANTES binding is displayed as a percentage of maximum specific binding. Inset, Scatchard transformation of the data, calculation of which showed a receptor density of 47,000 receptors/cell with a KD of 3.0 nM. Data are from a representative experiment (duplicates ± SD) of two to four experiments.

b, Competition of 0.3 nM 125I-labeled guinea pig eotaxin (i) or 0.1 nM concentrations of 125I-labeled hMCP-3 (ii) and 125I-labeled hRANTES (iii) and 100 nM concentrations of the unlabeled chemokines indicated. After washing, the transfectant cell pellets were counted in a gamma counter; ligand binding is displayed as a percentage of maximum specific binding. i, Data are the means ± SEM of three or four experiments. ii and iii, Data are from a representative experiment (duplicates ± SD) of two to four experiments. c, Chemotactic responses of the guinea pig CCR3 transfectant cell line. 4B4 cell transfectants were placed in Transwell cell culture inserts and incubated at 37°C in 24-well plates containing buffer or varying concentrations of guinea pig, human, and murine eotaxin; hMCP-3; hMCP-4; and human IL-8 as indicated. The chemotactic responses of the transfectants in response to these chemokines were measured by flow cytometry. Guinea pig eotaxin alone induced a chemotactic response that was maximal at 100 nM. No other tested agonist induced a chemotactic response, and these symbols overlap on the baseline. Data shown are from a representative experiment ± SD, which was repeated three times.

CLONING AND BLOCKADE IN VIVO OF GUINEA PIG CCR3

Stable guinea pig CCR3 transfectants were generated and assessed for their ability to migrate in response to guinea pig eotaxin. Two cell lines showing robust chemotaxis to guinea pig eotaxin (2A1 and 4B4) were further characterized for their ability to bind radiolabeled chemokines. Cell lines 2A1 and 4B4 showed identical ligand binding profiles, and therefore only data from the 4B4 line are shown here.

The 4B4 cells bound 125I-labeled guinea pig eotaxin (Fig. 2a), which was displaced by unlabeled guinea pig eotaxin in a concentration-dependent fashion. In previous studies using guinea pig eosinophils, binding of 125I-labeled guinea pig eotaxin was displaced both by hMCP-3 and hRANTES as well as guinea pig eotaxin (44). In keeping with these data, 4B4 cells, but not their untransfected parent cell line, also bound 125I-labeled hMCP-3 (Fig. 2, a and b) and 125I-labeled hRANTES (Fig. 2b). Scatchard analysis of the binding data indicated dissociation constants (KD) of 3.0 nM for hMCP-3 (Fig. 2a) and 4.5 nM for guinea pig eotaxin (mean of three experiments; data not shown), with a mean receptor density of 47,000 receptors per cell (Fig. 2a).

Binding of 125I-labeled guinea pig eotaxin, 125I-labeled hMCP-3, and 125I-labeled hRANTES was displaced by an excess of unlabeled guinea pig eotaxin or hMCP-3 (Fig. 2b). hRANTES failed to compete for 125I-labeled guinea pig eotaxin or 125I-labeled hMCP-3 binding in these experiments using the buffers described. However, when 125I-labeled hMCP-3 binding studies were performed in the RPMI buffer used in transfectant cell chemotaxis assays, 100 nM hRANTES competed for 53% of 0.1 nM 125I-labeled hMCP-3 binding to 4B4 cells (data not shown).

To evaluate ligand specificity further, we tested the chemotactic responses of the 4B4 cell line to the chemokines used in the radiolabeled ligand binding assays. Human IL-8 was included as a negative control. Untransfected L1.2 cells were unresponsive to all chemokines tested (Ref. 27 and data not shown). Fig. 2c shows that by increasing concentrations of unlabeled ligand. Inset, Scatchard transformation of the data, calculation of which showed a receptor density of 47,000 receptors/cell with a KD of 3.0 nM. Data are from a representative experiment (duplicates ± SD) of two to four experiments. b, Competition of binding of 125I-labeled guinea pig eotaxin, 125I-labeled hMCP-3, and 125I-labeled hRANTES to 4B4 cell transfectants. Transfectants were incubated with 0.3 nM 125I-labeled guinea pig eotaxin (i) or 0.1 nM concentrations of 125I-labeled hMCP-3 (ii) and 125I-labeled hRANTES (iii) and 100 nM concentrations of the unlabeled chemokines indicated. After washing, the transfectant cell pellets were counted in a gamma counter; ligand binding is displayed as a percentage of maximum specific binding. i, Data are the means from three or four experiments ± SEM. ii and iii, Data are from a representative experiment (duplicates ± SD) of two to four experiments. c, Chemotactic responses of the guinea pig CCR3 transfectant cell line. 4B4 cell transfectants were placed in Transwell cell culture inserts and incubated at 37°C in 24-well plates containing buffer or varying concentrations of guinea pig, human, and murine eotaxin; hMCP-3; hMCP-4; and human IL-8 as indicated. The chemotactic responses of the transfectants in response to these chemokines were measured by flow cytometry. Guinea pig eotaxin alone induced a chemotactic response that was maximal at 100 nM. No other tested agonist induced a chemotactic response, and these symbols overlap on the baseline. Data shown are from a representative experiment ± SD, which was repeated three times.

Construction of guinea pig CCR3 stable transfectants and characterization of receptor ligand binding

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Development of mAbs to guinea pig CCR3

Previous studies with a blocking mAb to human CCR3 have demonstrated the importance of this receptor in chemokine-induced chemotactic responses of eosinophils in vitro (12). This mAb does not cross-react with guinea pig CCR3 on either transfectants or guinea pig eosinophils, and therefore a panel of anti-guinea pig CCR3 mAbs was generated to explore the in vivo relevance of guinea pig CCR3 in well characterized guinea pig models of allergic inflammation (6, 7, 10). Following the first fusion, 1000 hybridomas were generated, and two Abs, 1C3 and 2A8, were isolated. Both mAbs bound to guinea pig CCR3 transfectants and showed minor cross-reactivity with the human CCR3 transfectant cell line (data not shown). Neither Ab stained a murine CCR3 transfectant or any other human CCR (1, 2, 4, and 5) or CXCR (1–3) transfectant tested (data not shown). The specificity of Abs 1C3 and 2A8 for guinea pig leukocytes was therefore investigated, and Fig. 3 shows that these Abs bound selectively to guinea pig eosinophils in PMNL populations purified from blood. There was no detectable binding of either 1C3 or 2A8 to guinea pig blood neutrophils (Fig. 3), monocytes, or lymphocytes as determined by flow cytometry (data not shown), in keeping with the previously described expression patterns of human CCR3 (27). A second fusion and round of hybridoma formation resulted in the identification of four further anti-guinea pig CCR3 mAbs with binding characteristics identical to those of 1C3 and 2A8 (data not shown).

Blockade of ligand binding and chemotactic responses of guinea pig CCR3 transfectants by anti-guinea pig CCR3 mAbs

To investigate the potential of the anti-guinea pig CCR3 mAbs to block ligand binding, the 4B4 cell line stable guinea pig CCR3 transfectants were pretreated with mAb 1C3 or 2A8, and the subsequent binding of 125I-labeled hMCP-3 was determined. Pretreatment of transfectants with mAb 2A8 blocked 125I-labeled hMCP-3 binding to transfectants in a concentration-dependent manner; in contrast, mAb 1C3 did not inhibit ligand binding at any concentration tested (Fig. 4). The binding characteristics of guinea pig CCR3 deduced from Fig. 2, a and b, and Ref. 44 predict that guinea pig eotaxin and hMCP-3 share a common binding site on guinea pig CCR3. Accordingly, pretreatment with 2A8 also blocked chemotaxis of the guinea pig CCR3 transfectants to guinea pig eotaxin in a concentration-dependent manner, at similar concentrations to those needed to inhibit 125I-labeled hMCP-3 binding (Fig. 4). Pretreatment of cells with the mAb 1C3 did not inhibit chemotaxis.

Blockade of guinea pig eotaxin signaling on guinea pig eosinophils by mAb 2A8

mAb 2A8 bound specifically to both guinea pig CCR3 transfectants and guinea pig eosinophils and blocked both ligand binding and chemotactic responses of guinea pig CCR3 transfectants. Therefore, the ability of 2A8 to block the in vitro responses of guinea pig eosinophils to guinea pig eotaxin was investigated using assays of chemotaxis and intracellular calcium flux. Pretreatment with 2A8 (10 μg/ml) of PMNLs purified from the blood of IL-5-treated guinea pigs caused a potent inhibition of eosinophil

FIGURE 3. Binding of anti-guinea pig CCR3 mAbs 1C3 and 2A8 to guinea pig leukocytes. Guinea pig PMNLs (comprising eosinophils and neutrophils) were incubated with purified 1C3 and 2A8 IgG (10 μg/ml). The cells were washed and incubated with FITC-conjugated goat antimouse secondary Abs. Following one further wash, the presence of bound Ab was detected by flow cytometry. A, Separation of guinea pig eosinophils and neutrophils in PMNL populations according to their light-scatter characteristics on FACS analysis. Both Abs bound to eosinophils (B), and neither Ab bound to neutrophils (C).
chemotaxis in response to guinea pig eotaxin but had no effect on the eosinophil chemotactic response to rhC5a (Fig. 5). In the same cell populations, guinea pig neutrophils showed a chemotactic response to rhC5a, but not to guinea pig eotaxin, and this was not inhibited by 2A8 pretreatment (data not shown).

To investigate further the blocking efficacy of 2A8 on guinea pig CCR3-mediated signaling in guinea pig eosinophils, elicited eosinophils were loaded with fura 2, and their intracellular calcium responses to guinea pig eotaxin were determined in the presence of buffer, 2A8 (12 μg/ml), and 1C3 (12 μg/ml). mAb 2A8 completely inhibited the guinea pig eosinophil calcium response to eotaxin.

### FIGURE 5.
Inhibition of guinea pig eosinophil chemotactic and cytosolic calcium responses to eotaxin by pretreatment with mAb 2A8. Guinea pig PMNLs purified from the blood of IL-5-treated donor animals were pretreated for 10 min with 2A8 (10 μg/ml) or buffer and were placed in Transwell cell culture inserts, which were incubated for 1 h at 37°C in 24-well plates containing guinea pig eotaxin (30 nM) or rhC5a (3 nM). The chemotactic responses of the leukocytes in response to these agonists were measured by flow cytometry (top). Pretreatment of the cells with 2A8 inhibited the chemotactic response to guinea pig eotaxin (*p < 0.05, ANOVA and Bonferroni’s post test) but not to rhC5a. Data shown are the means of four experiments ± SEM. In the lower panels, purified eosinophils were loaded with fura 2, and their intracellular calcium responses to guinea pig eotaxin were determined in the presence of buffer, 2A8 (12 μg/ml), and 1C3 (12 μg/ml). mAb 2A8 completely inhibited the guinea pig eosinophil calcium response to eotaxin.

Blockade of guinea pig eosinophil CCR3 signaling by F(ab’)2 fragments of mAb 2A8

In pilot experiments, i.v. administration of mAb 2A8 (1 mg/kg) to guinea pigs caused a rapid clearance of eosinophils from the circulation (data not shown), possibly mediated through nonspecific interactions of the Fc portion of the mAb. Therefore, for the use of 2A8 as a blocking anti-guinea pig CCR3 Ab in vivo, F(ab’)2 fragments of 2A8 were prepared. Fig. 6 shows that 2A8 F(ab’)2 fragments (30 μg/ml) bound to eosinophils similarly to whole Ab (10 μg/ml). 2A8 F(ab’)2 fragments (30 μg/ml) retained their ability to inhibit the eosinophil cytosolic calcium response to 3 nM guinea pig eotaxin but did not inhibit responses to rhC5a, used as a positive control for cell responses. Surprisingly, the results shown in Fig. 6 also suggest that guinea pig eotaxin partially desensitized the calcium response to rhC5a and that the blockade of eotaxin-induced responses by 2A8 pretreatment prevented this heterologous desensitization of the rhC5a response. Previous studies have found little
were labeled with $^{111}$In and pretreated with 100 µg/ml F(ab')$_2$ fragments of 2A8 or control Ab. After washing to remove unbound Ab, the cells were injected i.v. into eight recipients (four per Ab). Duplicate injections of buffer, guinea pig eotaxin, or LTB$_4$ were performed intradermally in the recipients in a randomized pattern. Two hours later, the animals were humanely killed, and the skin sites were punched out and counted in a gamma counter to determine the number of $^{111}$In-labeled eosinophils accumulating per skin site. * signifies $p < 0.05$ (significant difference between control and 2A8-pretreated cells; ANOVA and Bonferroni’s post test).

**FIGURE 7.** Inhibition of eosinophil accumulation in vivo by pretreatment with F(ab')$_2$ fragments of 2A8. Purified eosinophils were labeled with $^{111}$In and pretreated with 100 µg/ml F(ab')$_2$ fragments of 2A8 or control Ab. After washing to remove unbound Ab, the cells were injected i.v. into eight recipients (four per Ab). Duplicate injections of buffer, guinea pig eotaxin, or LTB$_4$ were performed intradermally in the recipients in a randomized pattern. Two hours later, the animals were humanely killed, and the skin sites were punched out and counted in a gamma counter to determine the number of $^{111}$In-labeled eosinophils accumulating per skin site. * signifies $p < 0.05$ (significant difference between control and 2A8-pretreated cells; ANOVA and Bonferroni’s post test).

**Discussion**

The potent eosinophil chemoattractant eotaxin was first isolated as a protein present in bronchoalveolar lavage fluid taken from a guinea pig model of allergic inflammation (6, 10). More recent studies in mice and humans have emphasized the role of this C-C chemokine, acting specifically on the CCR3 receptor, in mediating eosinophil accumulation (11, 16, 19, 21, 22, 30). CCR3 also functions as a coreceptor for HIV and may be particularly important in aspects of the pathology of AIDS such as HIV encephalopathy (47, 48). To understand further the roles of eotaxin and its receptor CCR3 in allergic inflammation, we have cloned and characterized guinea pig CCR3 and produced blocking mAbs that are effective in vivo.

Simultaneous screening of a guinea pig genomic DNA library with human and murine CCR3 cDNA probes identified a clone with homology to both these sequences. The DNA contained a single exon open reading frame coding for a G protein-coupled receptor with highest homology to human and murine CCR3 (67 and 69%, respectively).

When stably expressed in L1.2 cells, the receptor bound $^{125}$I-labeled guinea pig eotaxin with high affinity ($K_a$ 4.5 nM). The binding of radiolabeled ligand was effectively displaced both by unlabelled guinea pig eotaxin and hMCP-3. Human eotaxin was also able to displace 40% of the binding of $^{125}$I-labeled guinea pig eotaxin from the guinea pig CCR3 transfectants. The binding of $^{125}$I-labeled guinea pig eotaxin to guinea pig eosinophils has been shown to be competed by excess unlabeled guinea pig eotaxin, hMCP-3, and hRANTES (44). Indeed, hMCP-3 and hRANTES are antagonists of guinea pig eotaxin at this receptor in vitro, and hRANTES has been exploited as a prototypic receptor antagonist in vivo (44). In keeping with these data, the 4B4 cell stable guinea pig CCR3 transfectants also bound $^{125}$I-labeled hMCP-3 and $^{125}$I-labeled hRANTES. Binding of these ligands was effectively competed by an excess of unlabeled guinea pig eotaxin, indicating that they shared a common binding site on guinea pig CCR3. $^{125}$I-labeled hMCP-3 (available at a specific activity 11 times higher than that of $^{125}$I-labeled guinea pig eotaxin) was used to determine accurately guinea pig CCR3 expression levels on the transfectants and to verify the $K_a$. These studies demonstrated a mean receptor density on the 4B4 cells of 47,000 receptors per cell ($K_a$ 3.0 nM), in keeping with expression levels previously observed for other chemokine receptors using this expression system (27).

The binding of ligands to human CCR3 is very susceptible to minor variations in local buffer conditions (49). In our experiments, competition of ligand binding to stable guinea pig CCR3 transfectants (4B4 cells) was also partly influenced by the buffer (either a PBS-based or RPMI-based buffer) in which the binding studies were performed. In particular, competition of radiolabeled ligand binding to guinea pig CCR3 transfectants by unlabeled hRANTES was more dependent upon local conditions than the other ligands tested. $^{125}$I-labeled hRANTES bound to the 4B4 cells in both the PBS-based and the RPMI-based buffers (Fig. 2b and data not shown), and this binding was displaced by unlabeled guinea pig eotaxin, hRANTES, and hMCP-3. However, displacement of $^{125}$I-labeled guinea pig eotaxin and $^{125}$I-labeled hMCP-3 binding to the 4B4 cell line by unlabeled hRANTES was not observed (Fig. 2, a and b). When $^{125}$I-labeled hMCP-3 binding studies were then performed in the RPMI-based buffer (vs the PBS-based buffer used in the initial experiments), 53% displacement of $^{125}$I-labeled hMCP-3 by hRANTES was demonstrated. These variations in excess unlabeled hRANTES competition may also be partly explained by the tendency of this chemokine to become extensively aggregated at physiologic pH (50).

Of those ligands showing binding to guinea pig CCR3, only guinea pig eotaxin caused a functional response in the stably transfected 4B4 cell line. Chemotaxis, which was maximal in response to 100 nM guinea pig eotaxin, occurred over a concentration range similar to that seen with other L1.2 cell stable transfectants and their respective ligands (27). Guinea pig eotaxin is the only known native ligand for guinea pig CCR3. In particular, guinea pig RANTES has been cloned but has been shown to be active on guinea pig macrophages and not on guinea pig eosinophils (51).

To allow the investigation of the role of CCR3 and its ligands in vivo, we raised mAbs against guinea pig CCR3. From a panel of mAbs that bound to stable guinea pig CCR3 transfectants but not the parental L1.2 cell line, two (1C3 and 2A8) were chosen for...
further investigation. Both Abs bound selectively to guinea pig CCR3 transfectants; there was minimal cross-reactivity only with transfectants expressing human CCR3. These Abs also bound with high affinity to guinea pig eosinophils, but not monocytes, lymphocytes, or neutrophils, in keeping with the known expression patterns of CCR3 on human leukocytes (12). The mAb 2A8 inhibited both the binding of 125I-labeled hMCP-3 to stable transfectants and their chemotactic response to guinea pig eotaxin, and similarly inhibited the chemotactic and intracellular calcium responses of guinea pig eosinophils to guinea pig eotaxin.

In humans, there is evidence that T lymphocytes cultured in cytokines resulting in a Th2-type phenotype express CCR3 (35–38). CCR3+ lymphocytes in the circulation occur in very small numbers (35) and, even under stimulated Th2-type culture conditions, lymphocyte CCR3 expression may be at low levels compared with receptors such as CCR4 (37). It is possible that the numbers of T lymphocytes expressing CCR3 in either blood or bronchoalveolar lavage may be up-regulated in sensitized animals after allergen challenge, and this is a subject of further studies.

In preliminary experiments, we found that 2A8 IgG when injected i.v. into guinea pigs caused clearance of eosinophils from the circulation. Therefore, before in vivo investigation of the role of CCR3 by Ab-mediated receptor blockade, we prepared F(ab′)2 fragments of 2A8 to avoid possible Fc-mediated effects on eosinophil recruitment. These fragments retained their ability to block the guinea pig eosinophil responses to guinea pig eotaxin as determined in assays of intracellular calcium flux, with some reduction in potency, and had no effect on circulating eosinophil numbers.

To investigate the role of eosinophil CCR3 in the accumulation of eosinophils in response to chemokines, we pretreated 111In-labeled guinea pig eosinophils in vitro with control F(ab′)2 fragments or F(ab′)2 2A8 and examined their ability to accumulate in vivo in response to intradermal injections of guinea pig eotaxin and LTB4. Pretreatment of guinea pig eosinophils with F(ab′)2 2A8 selectively inhibited their accumulation in response to eotaxin but not to LTB4. Inhibition of eosinophil recruitment in response to intradermal eotaxin by F(ab′)2 2A8 was not complete. It is possible from the intradermal accumulation data alone to speculate the existence of a further functional guinea pig eosinophil receptor on guinea pig eosinophils that was not blocked by F(ab′)2 2A8 pretreatment. Insufficient F(ab′)2 2A8 was available to allow the co-administration of Ab i.v. with radiolabeled cells in sufficient concentration to block new or unblocked guinea pig CCR3 on circulating radiolabeled eosinophils and formally test this hypothesis. However, the existence of a second functional eosinophil receptor in the guinea pig would run counter to experience in all other species to date (52–54), and the in vitro data discussed above provide no support for the existence of a second functional eosinophil receptor on guinea pig eosinophils. This suggests that over the 2-h period during which the Ab-treated radiolabeled eosinophils circulated in vivo, there was either internalization of CCR3 with a subsequent reexpression of functional receptors not bound by 2A8 or some loss of F(ab′)2 2A8 from the cell surface.

In summary, we have cloned and sequenced the guinea pig eosinophil CCR3 receptor, guinea pig CCR3. We have characterized the ligand binding and signaling of this receptor and raised mAbs against it, one of which is a functional blocker of the receptor on guinea pig eosinophils in vitro. We have shown that pretreatment of guinea pig eosinophils with F(ab′)2 fragments of this Ab selectively inhibits eosinophil accumulation in response to guinea pig eotaxin in vivo, without affecting circulating eosinophil levels. Early evidence in vivo for the importance of chemotractant receptors in leukocyte recruitment was provided by studies demonstrating the inhibition of neutrophil recruitment by pretreating cells with pertussis toxin (55). More recent studies have demonstrated that blockade of eosinophil CCR3 in the guinea pig using hRANTES can inhibit eosinophil accumulation in vivo (44). Similarly, methionine-RANTES has been shown to inhibit eosinophil accumulation in mice (56). The present study is the first to show inhibition of eosinophil accumulation in vivo by a specific Ab directed against a chemokine receptor. Abs to chemokine receptors will be valuable tools to analyze the component parts of different types of inflammatory reactions. Such Abs may prove to be efficacious in certain inflammatory diseases in humans and provide proof-of-principle data for the development of small-molecule chemokine receptor antagonists.

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References


