Chemokine Receptor Usage by Human Eosinophils

The Importance of CCR3 Demonstrated Using an Antagonistic Monoclonal Antibody

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Abstract

Chemokines bind and signal through G-protein coupled seven transmembrane receptors. Various chemokine receptors are expressed on leukocytes, and these may impart selective homing of leukocyte subsets to sites of inflammation. Human eosinophils express the eotaxin receptor, CCR3, but respond to a variety of CC chemokines apart from eotaxin, including RANTES, monocyte chemotactic protein (MCP)-2, MCP-3, and MCP-4. Here we describe a mAb, 7B11, that is selective for CCR3 and has the properties of a true receptor antagonist. 7B11 blocked binding of various radiolabeled chemokines to either CCR3 transfectants, or eosinophils. Pretreatment of eosinophils with this mAb blocked chemotaxis and calcium flux induced by all CCR3 ligands. In all individuals examined, including allergic and eosinophilic donors, > 95% of the response of eosinophils to eotaxin, RANTES, MCP-2, MCP-3, and MCP-4 was shown to be mediated through CCR3. The IL-8 receptors, particularly CXCR2, were induced on IL-5 primed eosinophils, however these eosinophils responded to CC chemokines in the same manner as unprimed eosinophils. These results demonstrate the importance of CCR3 for eosinophil responses, and the feasibility of completely antagonizing this receptor. (*J. Clin. Invest.* 1997. 99:178–184.) Key words: chemokines • eosinophils • receptors-cytokine • cell movement • eotaxin

Introduction

Chemokines mediate a range of pro-inflammatory effects on leukocytes, including chemotaxis, degranulation, and integrin activation (1, 2). Numerous chemokines have been identified, and all bind to seven transmembrane spanning G-protein coupled receptors (7TMR*) (3–5). The best characterized chemokine receptors are the IL-8 receptor A (now termed CXCR1) which binds IL-8, and the IL-8 receptor B (CXCR2) which binds a number of the CXC or α chemokines, including IL-8 and GROα (3, 5). The known receptors for the CC or β chemokines include CCR1, which binds macrophage inflammatory protein (MIP)-1α,[1] RANTES and MCP-3 (4, 6–8), CCR2, which binds MCP-1 and MCP-3 (8, 9), CCR3, which binds eotaxin, RANTES and MCP-3 (10), CCR4 which binds MCP-1, MIP-1α, and RANTES (11, 12), and CCR5 which binds MIP-1α, MIP-1β, and RANTES (13–15). The expression of these receptors on different leukocyte subsets influences their migration to inflammatory sites. Neutrophils respond to many of the CXC chemokines (1), whereas T cells respond to the CC chemokines MCP-1, MCP-3, RANTES, MIP-1α, and MIP-1β (16–18), and the CXC chemokines IP-10, Mig, and SDF-1 (reviewed in reference 19).

Chemokine receptor usage by eosinophils has generated considerable interest, because eosinophils are selectively recruited to certain inflammatory sites (20, 21), and receptor antagonists may be useful for blocking eosinophil entry and degranulation in diseases such as asthma. Many chemokines have been reported to act on human eosinophils; RANTES, MCP-2, and MCP-3 were the first chemokines to be identified as eosinophil chemoattractants (22–24), followed recently by eotaxin (25) and MCP-4 (26). MIP-1α was found to be a weak chemoattractant in some studies (10, 23, 27), while IL-8 was found to attract eosinophils only after IL-5 stimulation (28). This array of ligands suggested a complex pattern of receptor expression on eosinophils. CCR1, the MIP-1α/RANTES receptor, was considered as a possible receptor on eosinophils for CC chemokines, but $[Ca^{2+}]_i$ desensitization and ligand binding studies implicated the existence of a distinct eosinophil receptor (22, 25, 29). CCR3, the eotaxin receptor, has subsequently been identified as a major CC chemokine receptor on eosinophils (10, 30). When transfected into a murine pre B lymphoma line, CCR3 bound eotaxin, RANTES and MCP-3, but not MIP-1α, and conferred chemotactic responses on these cells to eotaxin, RANTES and MCP-3 (10). The role of CCR1, CCR2, CCR4 and CCR5 on eosinophils is still unresolved, as is the receptor(s) on eosinophils used by MCP-2 and MCP-4.

Because of the complicated pattern of receptor binding and signaling by the chemokines, it has been difficult to determine the significance of a particular receptor on a given leukocyte type. In addition, Northern blot or ligand binding are sometimes not sensitive enough for detecting expression of chemokine receptors, which can be expressed at < 1,000 sites per cell (31). One way to address this is to develop specific receptor antagonists. Here we report on the first fully antagonistic mAb for a CC chemokine receptor. Anti-CCR3 mAb 7B11 completely blocks the binding and signaling of the known ligands

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1. *Abbreviations used in this paper:* MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein.

for CCR3. We show that human eosinophils use CCR3 and not other CC chemokine receptors, for their responses to eotaxin, RANTES, MCP-2, MCP-3 and MCP-4. Eosinophils activated in vitro with IL-5 showed the same properties, but upregulated CXCR1 and CXCR2.

Methods

Cells, cell lines, and tissue culture. Eosinophils were isolated from heparinized blood using CD16 microbeads (Miltenyi Biotec, Auburn, CA), as described (25) and were shown cytologically to be 99% pure. Neutrophils and PBMCs were isolated as described (25). To generate CD3 blasts, 2×10^6 PBMC/ml in RPMI-1640 plus 10% FCS were added to tissue culture plates first coated with the anti-CD3 antibody TR66. After 4–6 d blasts were removed to fresh media and supplemented with IL-2 (kindly provided by Antonio Lanzavecchia, Basel, Switzerland) at 50 U/ml. Other cell lines used included transfectants of the L1.2 murine pre B cell lymphoma, expressing high levels of either CCR3 (10), IL-8 RA (10), IL-8 RB (10), CCR2b (G. LaRosa, manuscript in preparation), CCR4 and CCR5 (Lijun Wu, manuscript in preparation), and CCR1 (32). Transfectants were maintained in RPMI-1640 supplemented with 10% bovine serum and 800 μg/ml G418. The different transfectants were monitored for expression of the relevant receptors, using mAbs specific for CCR3 (10), IL-8 RA, IL-8 RB, or CCR2 (16, 25). For CCR4 and CCR5, expression was monitored using the anti-flag mAb M2, since these receptors were constructed with this epitope at the NH_2 terminus. Human eosinophils were cultured with 5 ng/ml of recombinant human IL-5 (Genzyme Corp., Cambridge, MA), for 5–7 d, using tissue culture flasks containing sub confluent monolayers of ECV304 cells.

mAb production and flow cytometry. mAbs reactive with CCR3 were generated by immunizing C57BL6 mice with 10^7 L1.2 CCR3 transfected cells, intraperitoneally, five to six times at 2-wk intervals. The final immunization was injected intravenously. 4 d later, the spleen was removed and cells were fused with the SP2/0 cell line as described (33). mAbs reactive with CCR3 were identified using untransfected and CCR3 transfected L1.2 cells, and immunofluorescent staining and analysis using a FACScan® (Becton Dickinson & Co., Mountain View, CA). mAbs to IL-8 RA, IL-8 RB, and CCR2 (MCP-1R) have been described (16). mAb staining of cells was performed using standard procedures, as described previously (10). To enumerate antibody binding sites per cell, the following protocol was used: 100 μl of whole blood from donors was reacted with a supersaturating amount (400 ng) of a 7B11-FITC preparation in PBS with 0.5% azide at room temperature. Red cells were lysed with ammonium chloride lysing solution and the mean channel fluorescence of 7B11 stained cells was determined by flow cytometry. At the same time, the number of MESF units per antibody molecule was determined by saturating the sites on Simply Cellular beads (Flow Cytometry Standards Corp., San Juan, PR) with 7B11-FITC and reading the mean channel fluorescence of the beads on the flow cytometer. The flow cytometer was calibrated to equate mean channel numbers with MESF units using Quantum 26 beads (Flow Cytometry Standards Corp.). The number of antibody molecules per cell could then be calculated by calculating the MESF equivalents of the mean channel fluorescence of the cells and dividing by the MESF units per antibody molecule.

Chemokines, chemotaxis assays, and ligand-binding assay. Recombinant human chemokines were obtained from Peprotech (Rocky Hill, NJ), except for eotaxin, described previously (25), which was a gift of Dr. Ian Clark-Lewis. Chemotaxis of human eosinophils was assessed using a modification of a transendothelial assay (17), using the cell line ECV304 exactly as described (25). Cells that had migrated to the bottom chamber were placed in a tube, and relative cell counts were obtained using the FACScan®.

^{125}I-labeled eotaxin was obtained from Amersham Corp. (Arlington Heights, IL), and its specific activity was stated to be 2,000 Ci/mM. Chemokine binding to target cells was carried out as described previously (25, 34). Cells were washed once in PBS and resuspended in binding buffer (50 mM Hepes, pH 7.5, 1 mM CaCl, 5 mM $MgCl_2$, 0.5% BSA, and 0.05% azide) at a concentration of 10^7/ml. Aliquots of 50 μl (5×10^5 cells) were dispensed into microfuge tubes, followed by the addition of cold competitor and radiolabeled chemokines as indicated in the text. The final reaction volume was 200 μl. After a 60-min incubation at room temperature, the cells were washed three times with 1 ml of binding buffer containing 0.5 M NaCl. Cell pellets were then counted. The competition was presented as the percent specific binding as calculated by $100 \times [(S-B)/(T-B)]$, where S is the radioactivity of the sample, B is background binding, and T is total binding without competitors. Background binding was obtained by incubating cells with radiolabeled chemokine and at least 400-fold excess of unlabeled chemokines. Duplicates were used throughout the experiments and the standard deviations were always $< 10\%$ of the mean. All experiments were repeated at least three times. Curve fit and concentrations that inhibit 50% specific binding (IC50) were calculated by KaleidaGraph software (Synergy Software, Reading, PA).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$). A stock solution of Fura-2 AM (Molecular Probes, Eugene, OR) was prepared by dissolving 50 μg of the dye in 44 μl of DMSO. Immediately before addition to cells, this stock was diluted 1:100 into HBSS with Ca^{2+} and Mg^{2+} and 2% BSA. Fura-2 AM was added to cells at a final concentration of 0.2 moles/10^6 cells at 37°C for 30 min. After labeling, excess dye was removed by centrifugation and cells were resuspended at a concentration of 10^6/ml in 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM glucose, 0.025% BSA and 20 mM Hepes, pH 7.4. $[Ca^{2+}]_i$ was measured using excitation at 340 and 380 nm on a Hitachi F-2000 fluorescence spectrometer. Calibration was performed using 1% NP-40 for total release and 25 μM EGTA to chelate free Ca^{2+}.

Results

Complete blocking of eotaxin, RANTES, MCP-2 and MCP-3 binding to CCR3 transfectants using a mAb, 7B11. mAbs to CCR3 were generated, with the aim of developing a mAb that completely blocked CCR3 functions, so that the relevance of this receptor could be assessed. L1.2 transfectants expressing high levels of CCR3 (25) were used to immunize mice, and one mAb, 7B11, was identified that reacted with L1.2 cells transfected with CCR3, but not with L1.2 cells transfected with CCR1, CCR2b, CCR4, CCR5, CXCR1, or CXCR2 (Fig. 1 *A*). mAb 7B11 was found to be far superior to an anti-CCR3 peptide mAb, 5H12 (25), in that 7B11 stained human eosinophils intensely (Fig. 1 *B*). This mAb was unreactive with lymphocytes, CD3 activated T cells, and monocytes. Staining on neutrophils was largely negative, although a small percentage of these cells may express very low levels of the receptor. The small subset of granulocytes stained intensely by 7B11 (Fig. 1 *A*) were eosinophils which were contained in the granulocyte gate.

We tested mAb 7B11 for its ability to inhibit ^{125}I-labeled-eotaxin, -RANTES, -MCP-2, and -MCP-3 binding to CCR3 transfectants. mAb 7B11 completely inhibited binding of ^{125}I-labeled eotaxin to the transfectants (Fig. 1 *C*), and this inhibition was as efficient as that obtained with 100 nM cold eotaxin. This indicated that mAb 7B11 was able to completely block eotaxin binding to CCR3. This mAb also completely inhibited ^{125}I-labeled RANTES, ^{125}I-labeled MCP-3 and ^{125}I-labeled MCP-2 binding to CCR3 transfectants (Fig. 1 *C*), indicating that the epitope recognized by 7B11 was involved in the binding of numerous CC chemokines. However mAb 7B11 failed to inhibit RANTES binding to CCR1 transfectants (Fig. 1 *C*).

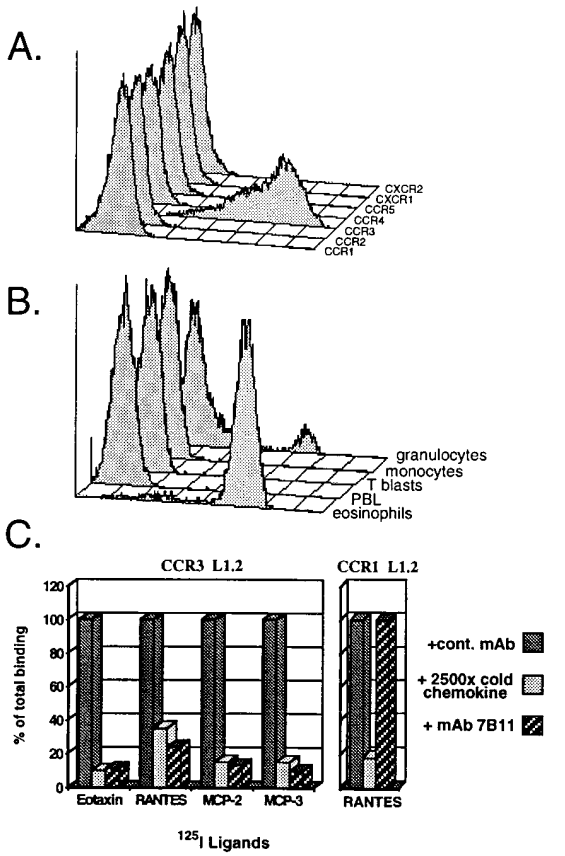

Figure 1. Identification of a neutralizing CCR3-specific mAb. (*A*) mAb 7B11 staining of various L1.2 transfectants. Stable L1.2 transfectants expressing either CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR1 (IL-8 RA), and CXCR2 (IL-8 RB) were stained with anti-CCR3 mAb 7B11. Negative control staining for all the L1.2 transfectants (not shown) resembled the staining shown for 7B11 on CCR1 transfectants. (*B*) Staining of human eosinophils, lymphocytes, T cell blasts, monocytes, and granulocytes with mAb 7B11. Staining profiles were representative of at least four experiments. (*C*) Binding of radiolabeled human eotaxin, RANTES, MCP-2, and MCP-3 to L1.2 CCR3 or CCR1 transfectants, and inhibition by mAb 7B11 or cold chemokines. Cells were incubated with 0.1 nM ^{125}I-labeled eotaxin, RANTES, or MCP-3, and either 50 μl of 100 μg/ml of irrelevant mAb (MOPC 21), mAb 7B11, or 250 nM cold chemokine. After 60 min at room temperature, cell pellets were washed and counted.

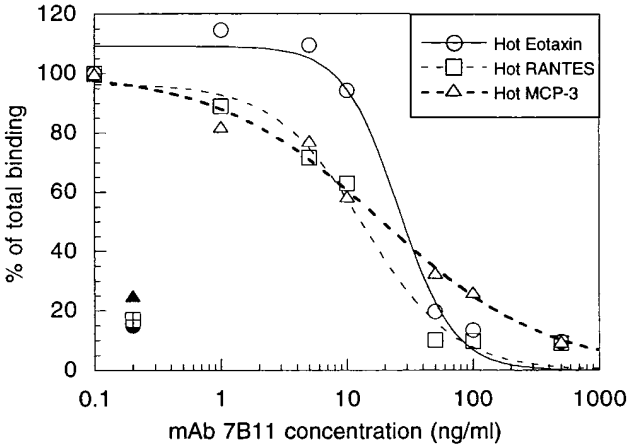

Figure 2. Inhibition of radiolabeled eotaxin, RANTES, and MCP-3 binding to human eosinophils by mAb 7B11. Human eosinophils were incubated with 0.1 nM ^{125}I-labeled-eotaxin, -RANTES, or -MCP-3, and various concentrations of mAb 7B11. After 60 min at room temperature, cell pellets were washed and counted. Data was analyzed by KaleidaGraph, which calculated an IC50 of eotaxin of 25.7 ng/ml, for RANTES of 13.7 ng/ml, and for MCP-3 of 18.8 ng/ml. The level of inhibition using 250 nM cold chemokine is shown at the bottom left of the plot: (●) eotaxin, (⊞) RANTES, and (▲) MCP-3.

mAb 7B11 blocks binding of radiolabeled eotaxin, RANTES and MCP-3 to eosinophils. To test if eotaxin, RANTES, and MCP-3 binding to eosinophils was occurring through CCR3, binding of radiolabeled chemokines to eosinophils was performed in the presence of various concentrations of the blocking mAb 7B11, or a control mAb (Fig. 2). ^{125}I-labeled eotaxin binding to eosinophils could be completely inhibited using an appropriate amount of 7B11 mAb, as expected, since eotaxin is known to bind only to CCR3 on eosinophils (10). However RANTES and MCP-3 are known to bind chemokine receptors in addition to CCR3 (4, 6, 10). Fig. 2 shows that mAb 7B11 also inhibited ^{125}I-labeled RANTES and MCP-3 binding to eosinophils. 50 ng/ml of mAb 7B11 was sufficient to achieve complete blockade of all chemokine binding to normal eosinophils, similar to the inhibition achieved with 2500-fold excess of cold chemokines. Slightly lower amounts of mAb 7B11 were required to block RANTES and MCP-3 binding, which is consistent with the lower affinity of RANTES and MCP-3 for CCR3 (10).

Inhibition of eosinophil chemotaxis to CC chemokines using anti-CCR3 mAb. Chemotaxis experiments were performed using eosinophils from normal individuals with moderately high levels of eosinophils (~ 3–6% of WBC). Fig. 3 *A* shows that mAb 7B11 was able to inhibit completely the chemotaxis of eosinophils to eotaxin in a dose dependent manner. 5–10 μg/ml was required to achieve 100% inhibition, using optimal concentrations of the various chemokines (usually 12.5 nM) in the bottom well. Fig. 3 *B* shows that the eosinophil chemotactic responses to RANTES, MCP-2, MCP-3, and MCP-4 could be inhibited totally using 5–10 μg/ml of mAb 7B11. 7B11 was unable to inhibit eosinophil chemotaxis to C5a (Fig. 3 *B*). Moreover, mAb 7B11 was unable to inhibit PBMC chemotaxis to RANTES (not shown), which occurs through chemokine receptors other than CCR3. We have observed donor to donor variation in eosinophil chemotactic responses to chemokines (10). Eosinophils from all individuals examined responded robustly to eotaxin, RANTES, MCP-2, MCP-3, and MCP-4, and mAb 7B11 was able to inhibit these responses by > 95%.

mAb 7B11 inhibits changes in $[Ca^{2+}]_i$ by eosinophils in response to CC chemokines. Eotaxin, RANTES, MCP-2, MCP-3 and MCP-4 induce changes in $[Ca^{2+}]_i$ by human eosinophils (25, 26). To examine the agonist/antagonist function of mAb 7B11, eosinophils were assessed for $[Ca^{2+}]_i$ after injection of mAb 7B11, or an irrelevant control mAb. Eosinophils incubated with the irrelevant mAb still produced changes in $[Ca^{2+}]_i$ after injection of optimal amounts of eotaxin, RANTES, MCP-2, MCP-3 and MCP-4 (Fig. 4, *top panels*). C5a, a potent stimulator of eosinophil $[Ca^{2+}]_i$, was used as a control.

Eosinophils incubated with 6.4 μg/ml of 7B11 mAb for 40 s were unable to respond to eotaxin, RANTES, MCP-2, MCP-3 or MCP-4 (Fig. 4, *bottom panels*). This inhibition was not due

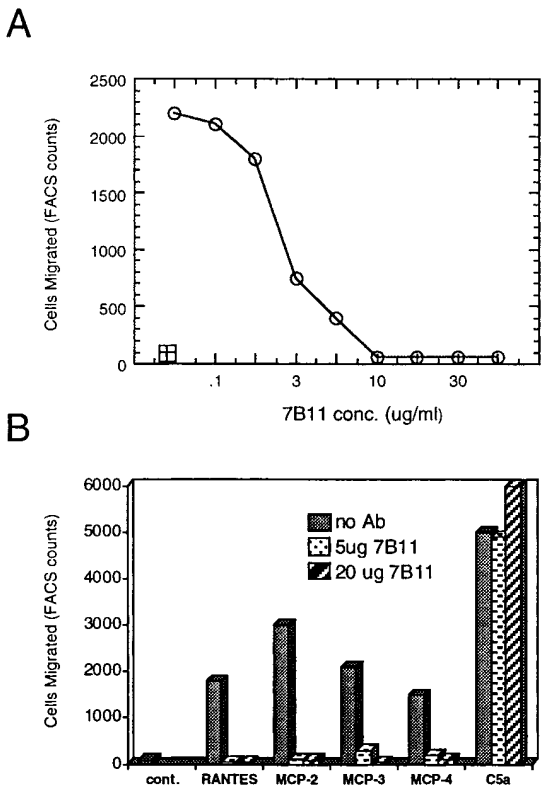

Figure 3. Inhibition of eosinophil chemotaxis to various chemokines by mAb 7B11. (*A*) Dose response of mAb 7B11 inhibition of eosinophil chemotaxis to eotaxin. The level of background migration of cells (no chemokine) is shown by the ⊞ symbol (bottom left of the plot). (*B*) Inhibition of eosinophil chemotaxis to various chemoattractants by 5 μg and 20 μg/ml of 7B11 mAb. For the experiments shown in both A and B, 1×10^6 human eosinophils were placed in the top chamber of the transwell and an optimal concentration of chemokine (usually 12.5 nM) was placed in the bottom chamber. Various concentrations of 7B11 mAb were placed in the top well. After 1.5 h the cells migrating to the bottom chamber were counted using flow cytometry. The results are representative of at least four separate experiments.

to receptor modulation from the cell surface, since this effect was rapid, and immunofluorescent staining of eosinophils incubated with mAb 7B11 at rt revealed intense staining (not shown). In addition, mAb 7B11 was antagonistic rather than agonistic, since concentrations as high as 10 μg/ml of mAb failed to induce a change in $[Ca^{2+}]_i$. 7B11 treated eosinophils showed no changes in $[Ca^{2+}]_i$ to C5a (Fig. 4). mAb 7B11 had no effect on the $[Ca^{2+}]_i$ of butyrate differentiated HL-60 cells to MIP-1α or RANTES, a response that is mediated through receptors other than CCR3 (not shown).

IL-5 primed eosinophils respond to CC chemokines through CCR3 but upregulate IL-8 receptors. Eosinophils from eosinophilic individuals, and normal eosinophils primed in vitro with IL-5, respond to IL-8 in chemotaxis assays (28, 35), suggesting that activated eosinophils have altered chemokine receptor expression. To test whether primed or activated eosinophils respond to CC chemokines in the same manner as do normal eosinophils, blocking experiments similar to those outlined in Figs. 3 and 4 were performed using day 5 to 7 IL-5 stimulated eosinophils, and eosinophils from an eosinophilic individual. The IL-8 receptors, CXCR1 and CXCR2, were undetectable by mAb staining on eosinophils from all normal individuals examined (n = 12) (Fig. 5 *A*). However after 5–7 d culture in vitro with human IL-5, CXCR2 and to a lesser degree CXCR1 were detectable on the surface of eosinophils, as detected using anti-CXCR2 mAbs and flow cytometry (Fig. 5 *B*), and this expression paralleled the ability of these eosinophils to migrate to IL-8 in chemotaxis assays (not shown). In the one eosinophilic donor examined (18–25% of WBC were eosinophils, for > 1 yr), CXCR2 was expressed on eosinophils at a slightly lower level (Fig. 5 *C*).

mAb 7B11 was still able to block completely the calcium responses of both IL-5 primed eosinophils (Fig. 5 *D*), and eosinophils from the eosinophilic donor (not shown), to eotaxin and RANTES (Fig. 5 *D*), as well as MCP-2, MCP-3, and MCP-4, in a similar fashion to that described for normal eosinophils. mAb 7B11 had no effect on IL-8 responses (Fig. 5 *D*), and MIP-1α responses were not evident in these experiments. CCR3 expression was assessed on the IL-5 primed eosinophils, and from eosinophils from numerous healthy individuals. The number of 7B11 binding sites per eosinophil from healthy individuals was calculated to be 17,400±1600 (n = 12), and no significant differences were observed after IL-5 stimulation. However in the one eosinophilic donor analyzed, the number of 7B11 binding sites was found to be 26,000.

Discussion

Here we showed that the functional effects of all of the efficacious chemokines for eosinophils-eotaxin, RANTES, MCP-2, MCP-3, or MCP-4, could be blocked completely with an anti-CCR3 mAb. This mAb was specific for CCR3, and had no inhibitory effects on other chemoattractant receptors. These results establish that CCR3 is indeed the principal receptor for eosinophil responses to CC chemokines, and questions an essential role for CCR1, CCR2, CCR4, or CCR5. These results also establish that the development of potent antagonists for the chemokine receptor family is viable, at least by the mAb approach.

CCR3 is expressed selectively and at high levels on eosinophils, as shown by ligand binding studies (10), and also staining with mAb 7B11. We also find expression of CCR3 on basophils and some T cell clones as well as tissue macrophages (unpublished observation). Before the identification of CCR3, there was indirect evidence for the expression of a MIP-1α/RANTES receptor on eosinophils, as well as a receptor with properties consistent with those of CCR3 (29). Also, a recent study suggested that human eosinophils express MIP-1α receptors, either CCR1, CCR4 or CCR5, at about 1–5% of the levels of CCR3 (27). We have been able to demonstrate modest eosinophil chemotactic responses towards MIP-1α in some individuals (10, 25), nevertheless our results show that a MIP-1α receptor contributes little to the functional responses of eosinophils to the major eosinophilic chemoattractants: RANTES, MCP-3 or MCP-4. We have observed donor to donor variation in eosinophil responses to the CC chemokines, however we were able to block these responses completely in all individuals, using mAb 7B11, suggesting that if other CC chemokine receptors are present, they have a minor functional significance. It is conceivable that inhibition of CCR3 somehow cross-inhibits the functions of other CC chemokine receptors,

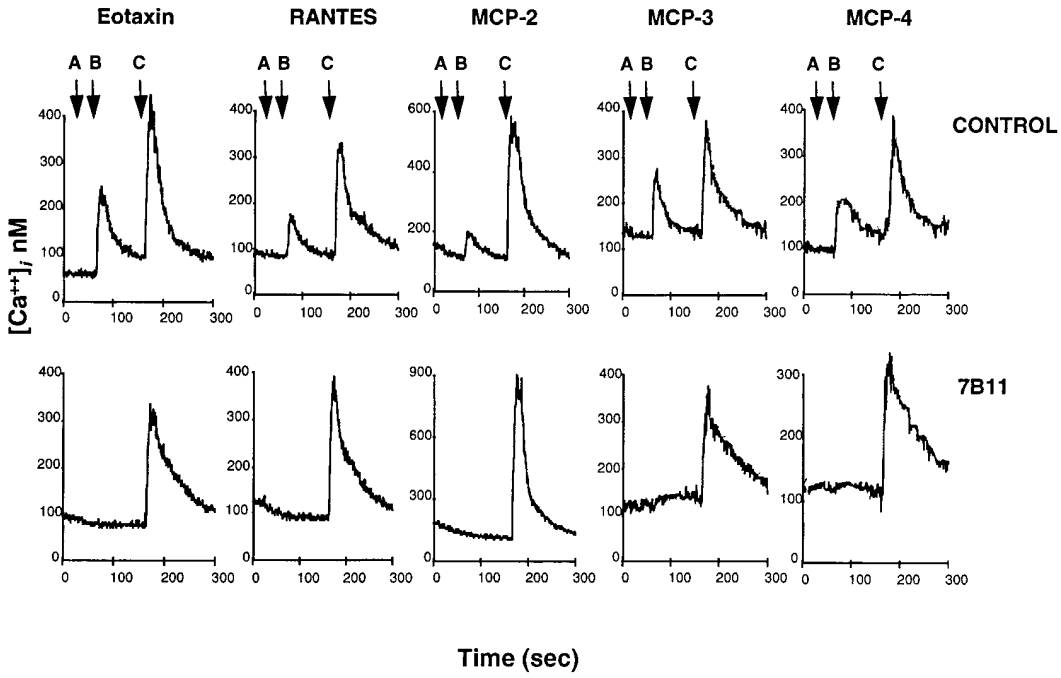

Figure 4. mAb 7B11 inhibits $[Ca^{2+}]_i$ by human eosinophils to eotaxin, RANTES, MCP-2, MCP-3 and MCP-4. Human eosinophils were labeled with Fura-2 as described in Methods, and stimulated sequentially with mAb (*A*), followed 40 s later with the indicated chemokine (*B*), and 100 s after that with C5a (*C*). $[Ca^{2+}]_i$ fluorescence changes were recorded using a spectrofluorimeter. The tracings were representative of five separate experiments, performed with eosinophils from different donors. In the top panels, an irrelevant mAb (MOPC-21) was used, and in the bottom panels, mAb 7B11. Antibodies were used at a final concentration of 6.4 μg/ml. Chemokines were used at: eotaxin, 10 nM, RANTES, 20 nM, MCP-2, 200 nM, MCP-3, 200 nM, MCP-4, 10 nM. C5a was used at 400 pM.

although this seems unlikely. We have observed that the MIP-1a/RANTES binding receptor, CCR5, is absent from the surface of eosinophils by mAb staining (C.R. Mackay, unpublished observation). Similar functional studies to those reported here using an antagonistic anti-CCR1 mAb will be important to define a role, if any, for CCR1 on eosinophils.

Eotaxin-CCR3 interaction is one mechanism that may explain the selective recruitment of eosinophils to sites of allergic inflammation, or parasitic infection (36, 37). CCR3 directed antagonists may therefore be ideal inhibitors of eosinophil recruitment to the airways, and thereby prevent the tissue damage mediated by eosinophilic granule proteins, a process which

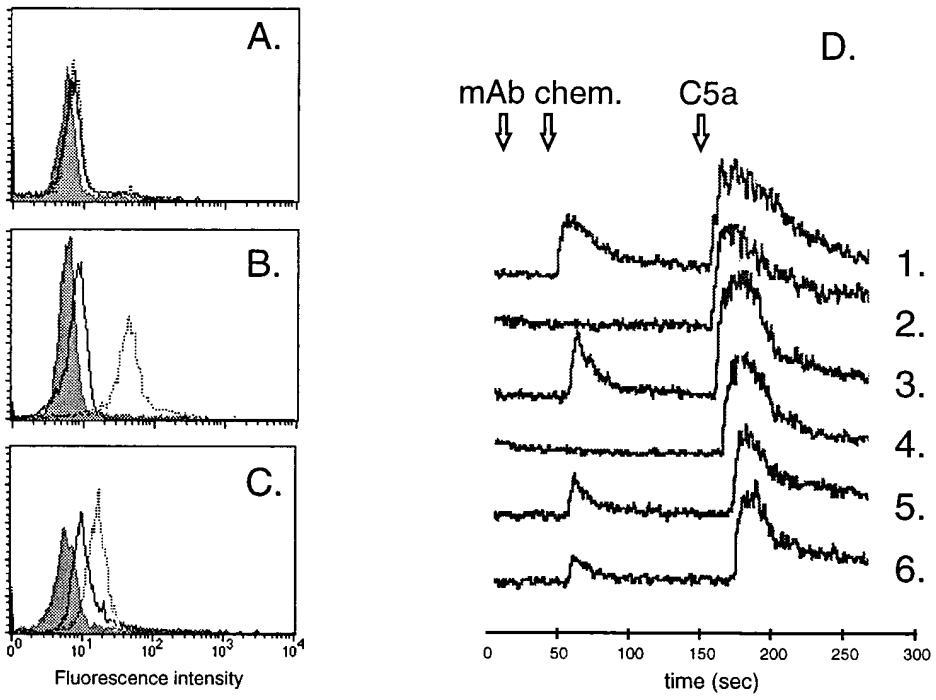

Figure 5. IL-5 primed eosinophils express IL-8 receptors, but respond to CC chemokines in a similar fashion to unprimed eosinophils. (*A*) IL-8 receptor expression on freshly isolated eosinophils from a healthy individual. Eosinophils were stained with mAbs to CXCR1 (*solid line*), CXCR2 (*dotted line*) or a control mAb (*shaded*), and were analyzed by flow cytometry. (*B*) IL-8 receptor expression on IL-5 treated eosinophils. Eosinophils cultured with IL-5 for 5 d were stained with mAbs, as in *A*. (*C*) IL-8 receptor expression on eosinophils isolated from an eosinophilic individual, and stained with mAbs, as in *A* and *B*. (*D*) Inhibition of $[Ca^{2+}]_i$ of day 5 IL-5 primed eosinophils to various chemokines by mAb 7B11. Methods were the same as those described in the legends of Fig. 4. The mAbs and chemokines used in the different tracings were: 1. control mAb, eotaxin, C5a; 2. 7B11, eotaxin, C5a; 3. control mAb, RANTES, C5a; 4. 7B11, RANTES, C5a; 5. control mAb, IL-8, C5a; 6. 7B11, IL-8, C5a. The results are representative of at least three separate experiments.

has been implicated in the pathogenesis of asthma (20). Blocking adhesion molecules such as β1 or β2 integrins is another strategy, and has proved effective in certain animal models (38, 39). Eosinophils are not known to express unique adhesion molecules, although they do differ from neutrophils in their expression of α4 integrins (40). The role of eotaxin-CCR3 in eosinophil migration to the airways in asthma is not yet proven, but is suggested since eotaxin and other chemokines are highly upregulated in animal models of allergic airway disease (41, 42). Chemokine receptor antagonists should be effective inhibitors of leukocyte recruitment, because the chemokines and their receptors appear to be fundamental for the recruitment process (43, 44), and neutralization of certain chemokines has proven effective at inhibiting a range of inflammatory reactions (44).

To our knowledge this is the first report of a fully antagonistic mAb to a CC chemokine receptor. We and others have produced antagonistic mAbs to CXCR1 and CXCR2 (16, 45, 46), however the ability of these mAbs to block ligand binding or signaling is less efficient than what we observed here for mAb 7B11 on eosinophils (unpublished). The C5a receptor has also been probed with antagonistic mAbs (47). The fact that there is one predominant chemokine receptor on human eosinophils, which binds many ligands, indicates that, for therapeutic applications, blocking this receptor will be more beneficial than blocking individual chemokines such as eotaxin or RANTES. The number of chemokines identified thus far numbers $\sim$ 40, and may end up exceeding 100. In addition to the five ligands identified so far, there could well be other ligands that bind CCR3, such as MCP-5, an eosinophil chemoattractant identified recently in the mouse (48). The chemoattractants that mediate aberrant eosinophil recruitment to the airways in asthma are not yet established, and it may be that different immune mechanisms, and different chemokines, operate in different individuals, making the blockade of a common receptor even more appealing.

Responses of IL-5–stimulated eosinophils to CC chemokines could also be blocked by mAb 7B11. We felt that this was important to test, because some chemokine receptors can be modulated after cytokine stimulation (49). In some hypereosinophilic individuals, eosinophils are activated, having a hypodense buoyant density and expressing markers associated with activation such as CD69 (50). IL-5, IL-3, or GM-CSF release is presumably responsible for this phenotype (50, 51). IL-5 did not have a dramatic effect on eotaxin or RANTES responsiveness by eosinophils, however it did lead to higher expression of IL-8 receptors. The relevance of IL-8 receptors on IL-5 primed or activated eosinophils is uncertain; our phenotypic and functional analyses are consistent with previous reports showing that IL-5 stimulated eosinophils, or eosinophils from eosinophilic donors, respond to IL-8 in chemotaxis assays (28, 35). Future studies should determine the role IL-8 receptors play in eosinophil chemotaxis in diseases such as asthma and hypereosinophilic syndrome, which are associated with high levels of activated eosinophils.

Acknowledgments

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