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Expression and characterization of the chemokine receptor CCR2B from rhesus monkey

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Abstract

Species selectivity of chemokine receptor antagonists is a potential deterrent to making preclinical assessments *in vivo*. To determine if rhesus monkey disease models could support these assessments, we pharmacologically and functionally characterized recombinant rhesus CCR2B receptor. For these studies we obtained the CCR2B coding region by PCR from genomic rhesus DNA and expressed the receptor as stable transfectants in Chinese Hamster Ovary cells. The surface expression of recombinant rhesus CCR2B was detected by flow cytometry using a commercially available monoclonal anti-hCCR2B antibody. This antibody was used to detect rhCCR2B on monocytes in peripheral blood mononuclear cell preparations from rhesus whole blood. The recombinantly expressed CCR2B exhibited similar high affinity binding to the CCR2 chemokine ligands from rhesus and human ^{125}I-rhMCP-1 ($K_d = 433 \pm 14$ pM) and ^{125}I-hMCP-1 ($K_d = 550 \pm 256$ pM). In competition binding, the receptor exhibited selective high affinity binding to the monocyte chemoattractant protein (MCP) family chemokines with little affinity for most other members of the CC family of chemokines. One exception was eotaxin, a high affinity ligand for CCR3, which bound to rhesus CCR2B receptor ($K_i = 1467 \pm 205$ pM). Chemokines which exhibited binding affinity for the receptor were tested for their ability to induce intracellular calcium release. In these experiments the relative potencies of the MCP family of chemokines for rhCCR2B were similar to the observed binding affinities. In contrast, eotaxin was functionally inactive as an antagonist or agonist to this receptor. TAK-799 (*N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride), a dual CCR2/CCR5 antagonist, demonstrated high affinity for the rhesus CCR2B in competition with ^{125}I-hMCP-1 binding to the receptor ($K_i = 0.5$ nM) and also potently blocked the MCP-1 induced calcium mobilization mediated through the receptor.

Keywords: CCR2B; Chemokine; Receptor; MCP-1; Rhesus monkey; Antagonist

1. Introduction

Chemokines are cytokines which are potent leukocyte chemoattractrants *in vitro*, and also appear to be responsible for controlling the migration of various leukocyte subsets *in vivo*. Most members of this family belong to one of two major subfamilies which are distinguished based on the arrangement of the first two conserved cysteines in the sequence: in the CC family the cysteines are adjacent to each other while in the CXC family they are separated by a single intervening amino acid. Chemokines exert their influence on cell migration through the activation of G protein-coupled receptors [1]. One member of this family, CCR2, is a key receptor for monocyte chemoattractants and is expressed on greater than 80% of circulating human monocytes [2]. Similar to many of the chemokine receptors, a number of high affinity ligands have been characterized for human and murine CCR2, all of which belong to the MCP subfamily (MCP-1 through MCP-4). Of these, MCP-1 (or CCL2) appears to be the most potent and CCR2

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Abbreviations: COPD, chronic obstructive pulmonary disease; EAE, experimental autoimmune encephalomyelitis; HIV, human immunodeficiency virus; FBS, fetal bovine serum; GF/B, glass-fiber filter; RANTES, regulated on activation normal T cell expressed and secreted; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; MIG, monokine induced by interferon-gamma or interferon-gamma-induced monokine.

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selective [3–5]. In addition, murine knockouts of CCR2 and MCP-1 highlight the critical and apparently non-redundant role this chemokine receptor/ligand pair plays in orchestrating the trafficking of monocytes under a number of inflammatory challenges *in vivo* [6–9].

Monocytes and monocyte-derived cytokines have been implicated in the pathogenesis of a number of autoimmune/inflammatory diseases, including rheumatoid arthritis [10,11], COPD [12,13], and atherosclerosis [14–16]. Because CCR2 plays a key role in the migration of blood borne monocytes to sites of inflammation [7–9], inhibition of monocyte recruitment, through CCR2, represents a potentially important therapeutic target for pharmacological intervention.

The rhesus monkey has served as a useful host for the development of animal models of both human immune-based diseases (like collagen-induced arthritis [17,18], EAE [19], and solid organ rejection [20,21]) and human infectious diseases (like HIV/SIV, anthrax [22], and lyme disease [23]). Frequently these models serve a vital role in the preclinical evaluation of therapies targeting these diseases, especially in situations in which the potential drugs demonstrate selective action in primates over rodents. In order to facilitate the investigation of the action of CCR2 antagonists in rhesus monkey disease models, we have characterized the binding and functional properties of recombinant and primary rhesus monkey CCR2 receptor. Additionally, the potency of TAK-779, one reported human CCR2/CCR5 dual antagonist [24], for the rhesus CCR2 receptor has been assessed.

2. Methods

2.1. Rhesus CCR2 DNA and sequence

The rhesus CCR2B (rhCCR2B) cDNA sequence deposited in GenBank in 1997 (Hauer *et al.*, GenBank accession # AF013958, GI: 2317747) [25] was used to design primers to amplify the coding region of this receptor (forward primer 5′-ATCGAAGCTTGCCACCATGCTGTCCACATCTCGTTCT-3′, reverse primer 5′-TTATAAACCAGCCGAGACTTC-3′). An update of this sequence (GenBank accession # AF013958, GI: 4206190) that modified the base 1073 from a C to a T which substituted valine for alanine at position 358 appeared after this project was initiated. The polymerase chain reaction (PCR) primers we used incorporated the original deposited sequence (Ala 358) at this position. A consensus Kozak (GCCACC) sequence [26] and a restriction site for *Hin*dIII was introduced in the forward primer to promote optimal expression and facilitate subsequent cloning steps. Since there are no introns in coding region of CCR2B in human or mouse, rhesus genomic DNA was used as a template for the PCR. PCR was performed using Platinum Taq DNA Polymerase High Fidelity (Life Technologies) according to manufacturer's directions. PCR cycling conditions were: 94° for 30 s; 55° for 30 s, and 68° for 70 s, repeated for 35 cycles. The resulting PCR product was cloned into vector pCR2.1 using a T/A cloning kit (Invitrogen). The sequence of two clones was determined by ACGT, Inc., using universal primers specific for the vector and additional primers based on the rhesus CCR2B sequence in GenBanK. Further digestion with restriction enzymes *Hin*dIII and *Not*I was then used to transfer the coding region to the mammalian expression vector pBJneo [27].

2.1.1. Transfection and cell selection

Chinese Hamster Ovary (CHO) cells were transfected with rhesus CCR2B plasmid DNA using LipofactAMINE (GIBCO-BRL). After transfection, cells were incubated for 3 days under geneticin (G418 sulfate) selection (1 mg/mL) and single cell clones were obtained by limiting dilution. Three clones were expanded and screened for binding to iodinated human MCP-1 (^{125}I-hMCP-1, PerkinElmer Life Sciences, Inc.) and the MCP-1 responsiveness in a calcium flux assay as described below. A single representative clone was used to characterize rhesus CCR2B in detail.

2.1.2. Cell isolation

(A) Rhesus peripheral blood mononuclear cells (PBMCs) were isolated from rhesus blood by layering the blood, after a 1:3 dilution with PBS, over Lymphocyte Separation Medium (LSM, ICN). The PBMC layer was collected after centrifugation at 1600 *g* for 20 min at room temperature, and residual red blood cells lysed with ammonium chloride solution (ACK, GIBCO-BRL). Cells were washed and resuspended in PBS. In general, the recovered cells using this method consisted of approximately 23% of monocytes. (B) T cell-depleted human PBMCs were isolated from venous whole or leukophoresed blood obtained from the Biological Specialty Corporation by Ficoll-Hypaque centrifugation and followed by rosetting with neuraminidase-treated sheep erythrocytes. This procedure routinely yields 70–90% monocytes as determined by morphology following cytospin dispersion and Wright–Giesma staining [28].

2.2. Flow cytometry

rhCCR2B-expressing CHO cells (10^6) were washed with 3 mL of staining buffer (PBS containing 5% FBS and 0.05% sodium azide). Cells were collected by centrifugation and the cell pellet was incubated with normal goat IgG on ice for 15 min prior to the addition of phycoerythrin (PE)-labeled mouse anti-hCCR2 monoclonal antibody (clone # 48607, R&D Systems) and followed by incubation for an additional 30 min. After staining, cells were washed once and resuspended in 0.3 mL of staining buffer containing 1 ug/mL propidium iodine. For staining of rhesus PBMCs, cells were washed once in staining buffer and then stained with α-CCR2, α-CD14 (Cat# 555397, Pharmingen), or relevant

isotype controls for 20 min at room temperature after blocking with normal goat IgG.

2.3. Direct radiolabeled binding assays

2.3.1. Equilibrium saturation studies

CHO cells (1×10^5) expressing rhCCR2B or human monocytes (2×10^5) were incubated with various amounts of either ^{125}I-hMCP-1 or ^{125}I-rhMCP-1 in the presence or absence of unlabeled MCP-1 in binding buffer for 60 min to reach equilibrium. The reaction was terminated by filtration of the reaction mixture through a GF/B filter plate. The plate was washed and dried and radioactivity was measured as described. The equilibrium binding constant (K_d) was obtained from the hyperbolic fit of a plot of the concentration of labeled ligand vs. the total radioactivity bound using Prism software (GraphPad).

2.3.2. Association binding

CHO cells (1×10^5) expressing rhCCR2B or human monocytes (2×10^5) were incubated with either ^{125}I-hMCP-1 or ^{125}I-rhMCP-1 at four different concentrations ranging from 0.5- to 4-fold the K_d value as determined by the previous saturation study. Each concentration of the ligand was incubated with cells for various times from 5 min to 2 hr in the presence or absence of unlabeled ligand. Bound radioactivity was determined as described above. For each ligand, the observed kinetic constant (k_{obs}) was determined from an exponential fit of the plot of specific binding with time for each concentration of ligand. The slope of a linear fit of ligand concentration vs. k_{obs} was used to determined the association constant (k_{on}).

2.3.3. Disassociation studies

CHO cells (1×10^5) expressing rhCCR2B or human monocytes (2×10^5) were incubated with either ^{125}I-hMCP-1 or ^{125}I-rhMCP-1 (at a concentration equivalent to the K_d) for 2 hr at room temperature to reach equilibrium. An excess (100 nM) of unlabeled hMCP-1 or rhMCP-1 was added to the reaction mixture to displace the ^{125}I-MCP-1 at various times and the samples harvested as described above. The dissociation rate and half-life of each ligand was evaluated from a single phase exponential decay fit of the total specific binding vs. time for each ligand.

2.4. Radioligand competition binding assays

CHO cells expressing rhesus CCR2B (5×10^4) or human monocytes (2×10^5) were incubated with ^{125}I-hMCP-1 (20–50 pM) or ^{125}I-rhMCP-1 (50–110 pM) and various concentrations of unlabeled chemokines (canine MCP-1 was custom synthesized by Gryphon Sciences, all other chemokines were obtained from Peprotech) in binding buffer for 60 min at room temperature. For antagonist binding experiments, unlabeled chemokine was replaced by antagonist diluted in DMSO. The same volume of DMSO was included in controls (final concentration of 2%). The binding buffer contains 50 mM HEPES, 5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4. ^{125}I-hMCP-1 was purchased from PerkinElmer Life Sciences, Inc., with specific activity of 2200 Ci/mmol and ^{125}I-rhMCP-1 (accession # AF255343) was synthesized by Gryphon Sciences and custom labeled with ^{125}I by PerkinElmer Life Sciences, Inc., to a specific activity of 824 Ci/mmol. The assay was terminated by filtration of the reaction mixture through GF/B filter plates (presoaked in 0.01% polyethyleneimine) using a Packard cell harvester. The filter plates were washed with 25 mM HEPES, pH 7.5, containing 500 mM NaCl (stored at 4°) and dried in incubator at 42°. The plates were loaded with Microscint 0 (Packard) and counted in a Topcount NXT (Packard). The Prism computer software program (GraphPad) was used to calculate the inhibition of radioactive ligand binding.

2.5. Functional assay for intracellular calcium release

CHO cells expressing rhCCR2B (4×10^4) were incubated in cell culture medium containing Fluo-3, AM fluorescent dye (5 ug/mL, Molecular Probes), and probenicid (710 ug/mL, Sigma) for 1 hr at 37°. Cells were washed with Hanks buffer containing HEPES (20 mM), BSA (0.1%), and probenicid, and then were resuspended in 90 uL of the same buffer. A ligand addition plate was prepared by adding 135 uL of chemokines at various concentrations for the titration. To test inhibition of calcium release by TAK-779, 2 uL of this antagonist at various concentrations was added to the cells upon resuspension and 20 nM hMCP-1 was used to activate CCR2B receptors. Both plates were placed into the Fluorescence Imaging Plate Reader (FLIPR, Coherent, Inc.). Chemokines were dispensed from addition plate into the cell plate and calcium release was measured by the Argon Laser at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

3. Results

3.1. Rhesus CCR2 receptor sequence and creation of stable cell lines

The rhesus CCR2B cDNA sequence encodes 360 amino acids and is 97% homologous to human CCR2B (Fig. 1) [25]. Based on sequence information deposited in GenBank, we designed PCR primers corresponding to nucleotides encoding amino acid residues 1–7 (MLSTSRS) and residues 356–360 (VSAGL) to amplify the coding region of this receptor. The sequences of two independent clones of the resultant PCR fragment were analyzed. In every instance the sequence we obtained for rhCCR2B contained a G at position 375 in contrast to the T in the original

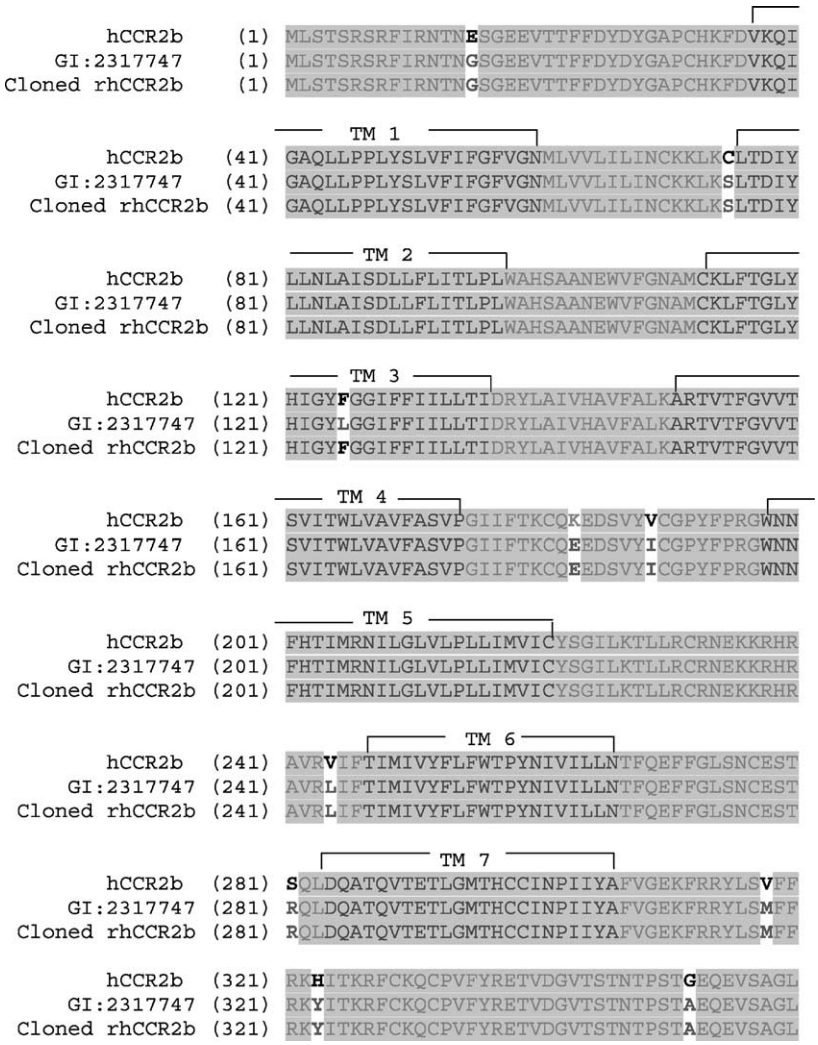

Fig. 1. Peptide sequences alignment of human CCR2B, rhesus CCR2B from GenBank (GI: 2317747), and the rhesus CCR2B sequence we obtained. Like human CCR2B, rhesus CCR2B encodes 360 amino acids and is 97% homologous to human CCR2B. Identical regions of the receptors are shown in shaded area. Transmembrane domains are indicated.

GenBank deposited sequence. This change results in a Phe at position 125 in place of leucine. Because this position is in a region of transmembrane domain 3 which is highly conserved in CCR2 among species (Fig. 2) and has been implicated in the binding of small molecule agonists and antagonists of other chemokine receptors and GPCRs [29–31], we performed a number of experiments to further confirm this initial result. Sequencing of the products of six independent PCR reactions using the same initial source of template rhesus genomic DNA as well as of independent PCR reactions using genomic DNA template isolated from five additional rhesus monkeys all confirmed the identity of the amino acid at position 125 as a phenylalanine.

The cloned rhCCR2B receptor was transfected into CHO cells and 47 neomycin-resistant single cell clones were isolated. Ten clones were chosen based on their ability to bind ^{125}I-hMCP-1 and further tested for the functional responsiveness to hMCP-1 in a calcium flux

Fig. 2. The sequence of rhesus CCR2B we obtained is different from original rhesus CCR2B sequence in GenBank (GI: 2317747) at residue 125. Our sequence contained phenylalanine in this position rather than the leucine in the published sequence. Alignment of all known CCR2 peptide sequences indicates phenylalanine at position 125 is highly conserved.

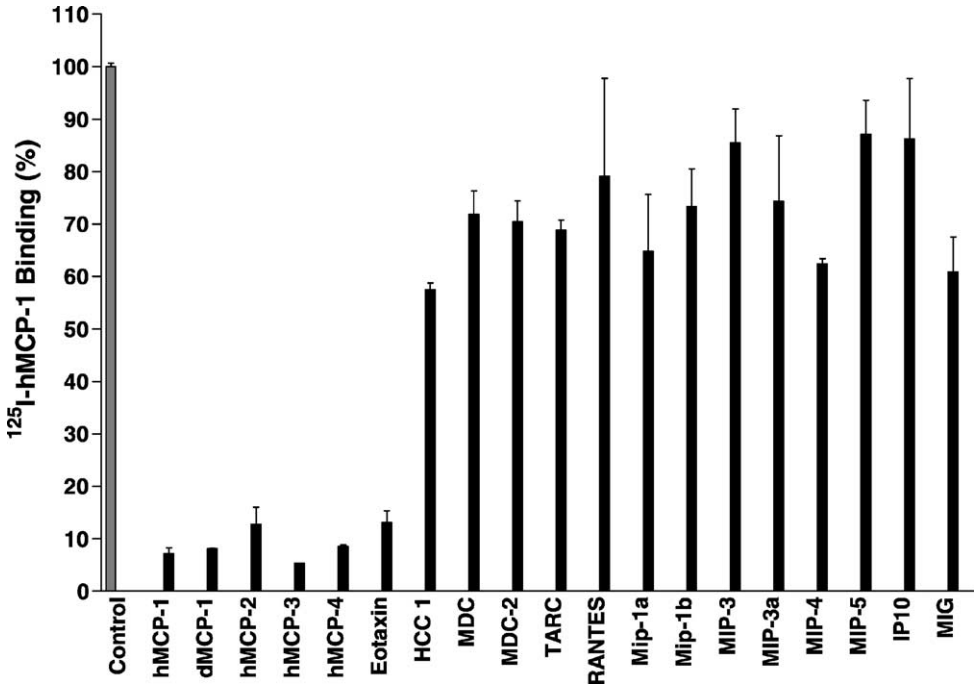

Fig. 3. Binding of various chemokines to cloned rhesus CCR2B receptor. One hundred nanomoles of each chemokine was used to compete with human ^{125}I-MCP-1 (25 pM) binding to the cloned receptor. Members of MCP family and eotaxin demonstrate significant binding affinity for this receptor (N = 2).

assay. A single representative clone, clone 12, which had intermediate levels of ^{125}I-hMCP-1 binding relative to the other clones obtained and which demonstrated a robust mobilization of intracellular calcium in response to MCP-1 stimulation was chosen for further studies.

3.2. Radiolabeled ligand binding studies

Competition binding studies were performed in order to characterize pharmacological properties of cloned rhCCR2B. Various chemokines of the CC family were tested for their ability to compete with commercially available ^{125}I-hMCP-1 binding to rhCCR2B transfected cell line clone 12. As shown in Fig. 3, members of MCP family hMCP-1, rhMCP-1, murine MCP-1 (not shown in graph), canine MCP-1 (dMCP-1), hMCP-2 (CCL8), hMCP-3 (CCL7), hMCP-4 (CCL13), as well as eotaxin (CCL11), a high affinity ligand for CCR3 [27], competed against specific rhCCR2-ligand binding at 100 nM. In contrast, a number of other human chemokines, including RANTES (CCL5), HCC1 (CCL14), TARC (CCL17), MDC (CCL22), MDC-2, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), MIP-3, MIP-3α (CCL20), MIP-4 (CCL26), MIP-5 (CCL15), and MIG (CXCL9), ligands for other chemokine receptors inhibited <60% of ^{125}I-hMCP-1 at this concentration.

To further characterize the pharmacology of the rhesus CCR2B receptor, the binding affinities between cloned receptor and all MCP family members and eotaxin, whose sequence is closely related to those of the MCP family chemokines, were further characterized by full competition binding experiments. As shown in Fig. 4c and d, when competing with ^{125}I-rhMCP-1, rhMCP-1, hMCP-1, canine MCP-1, and murine MCP-1 demonstrated high affinities (K_i's of 200–300 pM) for the cloned receptor while hMCP-3 and hMCP-4 had slightly reduced affinities (K_i's of ~600 pM), and hMCP-2 and eotaxin demonstrated 5- to 6-fold lower affinities (K_i's of >1 nM). The results were similar in experiments using ^{125}I-labeled hMCP-1. The relative affinities of various chemokines for rhCCR2 are similar to those obtained when using human monocytes as a source of human CCR2 (Fig. 4a).

The kinetic and equilibrium binding properties of radiolabeled rhMCP-1 and hMCP-1 were also studied using the cloned rhesus CCR2B and compared to ^{125}I-hMCP-1 on human monocytes (Table 1). Consistent with the competition binding experiments described above, ^{125}I-hMCP-1 and ^{125}I-rhMCP-1 have similar direct binding characteristics. Both the saturation equilibrium binding constant (K_d, 550 pM vs. 433 pM) and the kinetic binding properties of labeled human and rhesus MCP-1 to rhCCR2 are comparable; the on-rates (k_{on}) are very similar, while the off-rate (k_{off}) is 5.6×10^{-3} for hMCP-1 vs. 4.9×10^{-3} for rhMCP-1, corresponding to half-life values ($T_{1/2}$) of 126 and 136 min, respectively.

3.3. Functional activation of rhCCR2: intracellular Ca^{2+} flux

To verify that rhCCR2B was functionally coupled in CHO cells, changes of intracellular Ca^{2+} levels were measured in response to various chemokines. As shown

(A)

Chemokines	Rhesus CCR2B/CHO ^{125}I-hMCP-1 (n)	Rhesus CCR2B/CHO ^{125}I-rhMCP-1 (n)	Human Monocytes ^{125}I-hMCP-1 (n)
Rhesus MCP-1	228±47 (5)	267±58 (5)	749±337 (5)
Human MCP-1	323±74 (4)	280±36 (5)	847±382 (5)
Canine MCP-1	246±63 (5)	228±46 (5)	793±376 (5)
Murine MCP-1	212±51 (5)	220±52 (5)	458±132 (5)
Human MCP-2	1218±264 (5)	1342±162 (4)	1665±331 (5)
Human MCP-3	627±132 (5)	701±164 (5)	837±158 (5)
Human MCP-4	601±80 (5)	615±91 (5)	2571±831 (5)
Human Eotaxin	1467±205 (5)	1489±185 (5)	2102±492 (5)

(B)

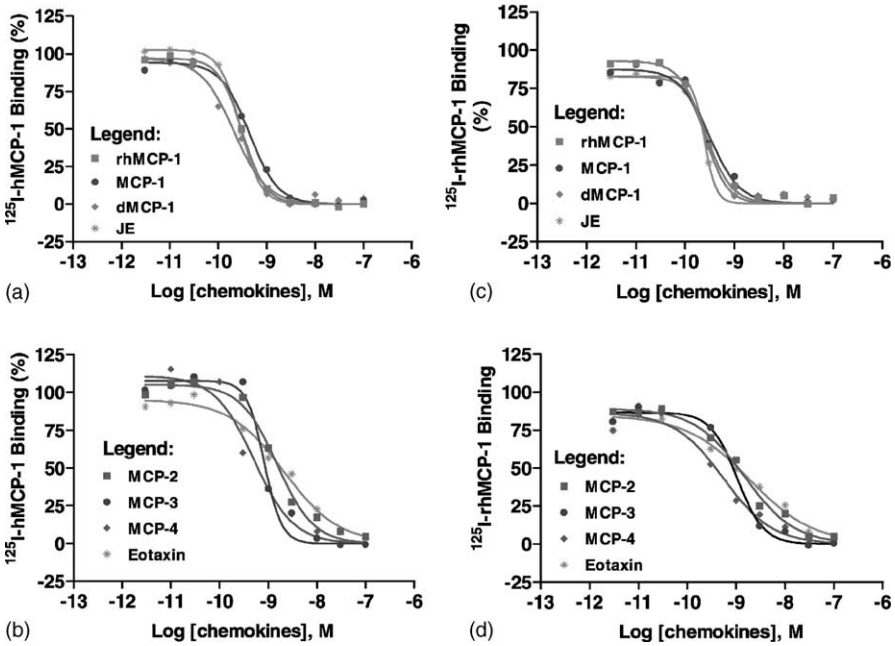

Fig. 4. Competition binding of chemokines against human ^{125}I-MCP-1 (B(a) and (b)) or rhesus ^{125}I-MCP-1 (B(c) and (d)). Results in the table (A) are the averages of several experiments (N ≥ 4).

in Fig. 5, rhMCP-1 and hMCP-1 induced Ca^{2+} flux in cells expressing the receptor with average EC_{50} of 4 nM. Canine and murine MCP-1 had similar potencies. Stimulation with MCP-2 gave a total response which was approximately 40% that observed with MCP-1 and a potency greater than 20 nM (data not shown). In contrast, MCP-3 and MCP-4 typically gave 70% the total response observed with MCP-1 and have EC_{50}'s of 15–20 nM (data not shown).

Table 1
Kinetic binding properties of hMCP-1 and rhMCP-1

	Saturation K_d (pM)	On-rate (pM^{-1} min^{-1})	Off-rate (min^{-1})	$T_{1/2}$ (min)
Rhesus CCR2/CHO				
^{125}I-hMCP-1[a]	550 ± 256 (2)	3.1×10^{-5} (1)	5.6×10^{-3} (2)	126 (2)
^{125}I-rhMCP-1[b]	433 ± 14 (2)[c]	3.4×10^{-5} (1)	4.9×10^{-3} (1)	136 (1)
Human monocytes				
^{125}I-h MCP-1[d]	132 ± 28 (4)	6.2×10^{-5} (1)	8.4×10^{-3} (1)	83 (1)

K_d values are means ± SD, the number of experiments is indicated in parentheses. Values for the other parameters are the average of the number of experiments indicated in parentheses.

[a] 50,000 rhesus CCR2/CHO cells plus human ^{125}I-MCP-1.

[b] 50,000 rhCCR2/CHO cells plus rhesus ^{125}I-MCP-1.

[c] K_d values are the average of two independent clones that express rhesus CCR2B.

[d] 200,000 human monocytes plus human ^{125}I-MCP-1.

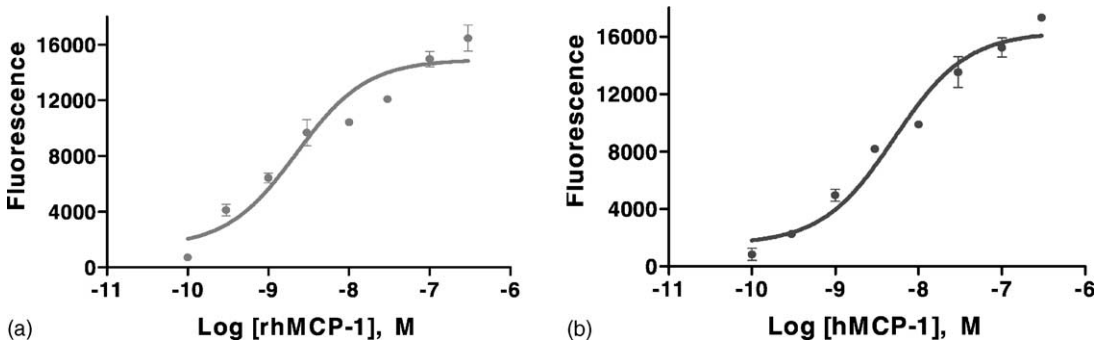

Fig. 5. Rhesus CCR2B-mediated Ca^{2+} flux in CHO cells. Rhesus MCP-1 (a) and human MCP-1 (b) can induce Ca^{2+} flux with similar potencies. These graphs are representative of multiple experiments.

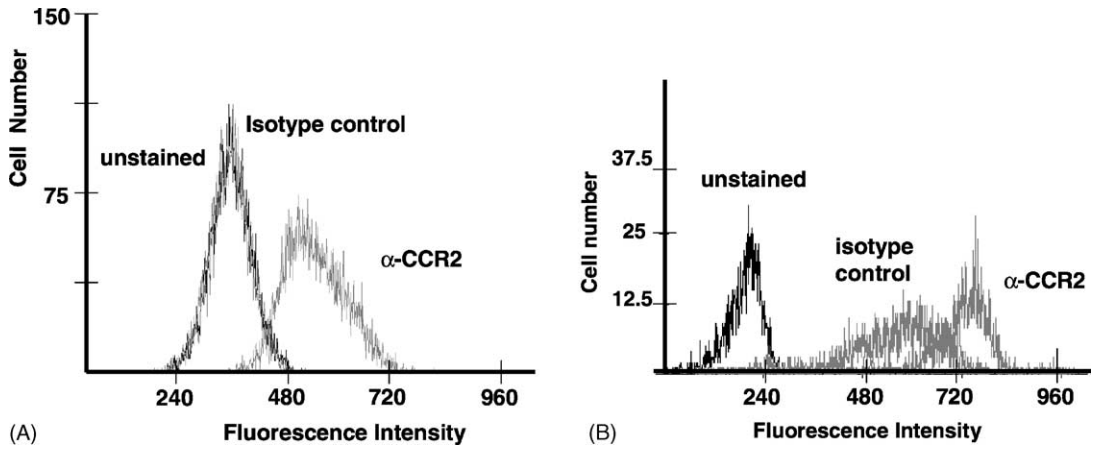

Fig. 6. Flow cytometry analysis of rhesus CCR2-expressing CHO cells (A) and rhesus peripheral blood monocytes (B). The latter were gated by forward and side scatter (CD14 positive population). PE-labeled mouse anti-human CCR2 monoclonal antibody or Ig2B isotype control (R&D Systems) was used.

3.4. Flow cytometry of rhCCR2

The cell surface expression of cloned rhCCR2B protein was analyzed by flow cytometry using a commercially available PE-labeled mouse anti-hCCR2 monoclonal antibody. Cell surface expression of CHO-rhCCR2B clone 12 was detected (Fig. 6A), indicative of the cross-reactivity of this antibody for the rhesus receptor. Flow cytometric analysis of rhesus PBMCs with this antibody demonstrated that >75% of rhesus monocytes (which were identified by forward and side scatter properties) expressed CCR2B (Fig. 6B).

3.5. Binding and functional block of rhCCR2 by a small molecule antagonist to rhCCR2

The binding of TAK-779, a small molecule hCCR2/hCCR5 dual antagonist, was tested in a standard ^{125}I-hMCP-1 competition binding assay. This compound had excellent affinity for the receptor, with a K_i of 0.5 nM (Fig. 7A). In addition, TAK-779 was shown to be a potent functional antagonist for the rhesus CCR2B *via* inhibition of hMCP-1 induced calcium flux with an IC_{50} of 1 nM (Fig. 7B).

4. Discussion

Using primers based on the initially deposited sequence for CCR2B (GenBank GI: 2317747), we have independently obtained a sequence for the rhesus CCR2B receptor which differs by one amino acid; the leucine at position 125 is replaced with a Phe in our sequence (Fig. 1). In all other known CCR2B sequences from different species Phe 125 is highly conserved (Fig. 2). Although we cannot rule out the possibility that this change represents a true polymorphism at this locus, our data indicate that phenylalanine is the amino acid found at position 125 in most rhesus individuals; six of six rhesus tested had this sequence.

Many chemokine receptors bind to and are activated by more than one chemokines, and the repertoire of chemokines active at a particular receptor shows some species variability. Previous work to date in both the mouse and human CCR2 receptors has suggested that MCP-1 is unique in its affinity for CCR2 among identified chemokine receptors, while other chemokines, including other members of the monocyte chemoattractant family (MCP family), have affinity at multiple chemokine receptors [1,32]. Nearly half of the amino acid differences (4/10) between the rhesus and human CCR2B sequences are in

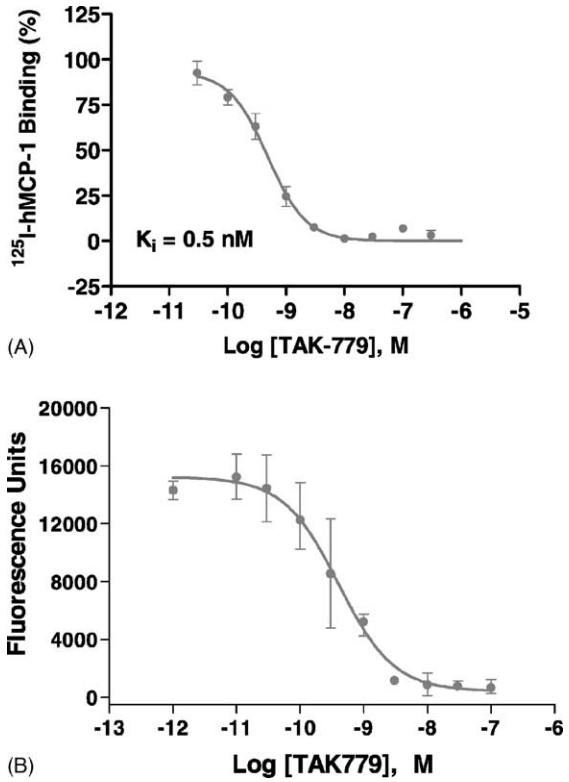

Fig. 7. (A) Competition binding of TAK-779 against ^{125}I-hMCP-1 to rhesus CCR2B-expressing CHO cells. $K_i = 0.5$ nM (N = 1). (B) Inhibition of hMCP-1-induced Ca^{2+} flux in rhesus CCR2B-expressing CHO cells ($IC_{50} = 0.8\,nM \pm 0.5$, average of two experiments ± range).

either the N-terminal tail or the second extracellular loop of the protein both of which are regions of the protein proposed to interact with chemokine ligands [33]. Therefore, the equilibrium and kinetic binding parameters of both human ^{125}I-MCP-1 and rhesus ^{125}I-MCP-1 to rhesus CCR2 were determined. These experiments demonstrate that both ligands bound to the rhesus receptor with similar equilibrium and kinetic constants. Similar direct binding parameters were also obtained for the binding of ^{125}I-hMCP-1 to human monocytes. The hMCP-1 and rhMCP-1 peptide sequences are highly homologous and differ in only two amino acid residues in the secreted protein, which do not appear to influence the affinity for the rhesus receptor (see Fig. 8).

In addition, competition experiments using both human ^{125}I-MCP-1 and rhesus ^{125}I-MCP-1 were used to determine the ligand specificity of the recombinant rhesus CCR2 receptor for other chemokines. Similar results were obtained independent of which radiolabeled ligand was used. In these experiments, the binding affinities for various chemokines were ranked as follows: rhesus, human, dog, and murine MCP-1 > MCP-3 and MCP-4 > MCP-2 and eotaxin. In contrast, RANTES, HCC1, TARC, MCD, MCD-2, MIP-1α, MIP-1β, MIP-3, MIP-3α, MIP-4, MIP-5, and MIG had weak affinities for rhCCR2 (i.e. $IC_{50} > 100$ nM). These results were further confirmed using a different rhesus CCR2B-expressing CHO cell clone and using PBMCs from two non-related rhesus

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 human MCP-1     (1) QPDAINAPVTCCYNFTNRKISVQRLASYRR
rhesus MCP-1     (1) QPDAINAPVTCCYNFTNRKISVQRLASYRR
canine MCP-1     (1) QPDAIISPVTCCYTLTNKKISIQRLASYKR
murine MCP-1     (1) QPDAVNAPLTCCYSFTSKMIPMSRLESYKR

 human MCP-1    (31) ITSSKCPKEAVIFKTIVAKEICADPKQKWV
rhesus MCP-1    (31) ITSSKCPKEAVIFKTIVAKEICADPKQKWV
canine MCP-1    (31) VTSSKCPKEAVIFKTVLNKEICADPKQKWV
murine MCP-1    (31) ITSSRCPKEAVVFVTKLKREVCADPKKEWV

 human MCP-1    (61) QDSMDHLDKQTQTPKT--------------
rhesus MCP-1    (61) QDSMDHLDKQIQTPKP--------------
canine MCP-1    (61) QDSMAHLDKKSQTQTAKP------------
murine MCP-1    (61) QTYIKNLDRNQMRSEPTTLFKTASALRSSA


 human MCP-1    (77) ------------------------------
rhesus MCP-1    (77) ------------------------------
canine MCP-1    (79) ------------------------------
murine MCP-1    (91) PLNVKLTRKSEANASTTFSTTTSSTSVGVT

 human MCP-1    (77) -----
rhesus MCP-1    (77) -----
canine MCP-1    (79) -----

murine MCP-1   (121) SVTVN
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Fig. 8. Comparison of MCP-1 sequences for different species. Only the sequence after the cleaved signal peptide is shown. The differences between human and rhesus MCP-1 are highlighted in bold. Accession numbers for sequences: human MCP-1 (P13500), rhesus MCP-1 (Q9MYN4), canine MCP-1 (P52203), murine MCP-1 (P10148).

monkey donors (not shown). These relative affinities are comparable to what has been observed for human CCR2 (human monocytes), with MCP-1 possessing the highest affinity followed by other members of the MCP family [3,4,34]. Also similar to what has been observed for both mouse and human CCR2, other members of the CC-chemokine family do not have significant affinity for rhCCR2 [5,35]. Since ^{125}I-rhMCP-1 and ^{125}I-hMCP-1 yielded similar results in both direct binding assays as well as in competition studies (Table 1 and Fig. 4), the more readily obtainable and commercially available hMCP-1 was used in all subsequent studies.

Chemokine receptors, including CCR2, typically couple to heterotrimeric G proteins of the G_i family leading to downstream signaling events that include mobilization of intracellular calcium. The rise in intracellular calcium levels upon stimulation with various chemokine ligands was therefore monitored using an intracellular calcium reporter dye system. The results of these experiments demonstrated that the binding of selective chemokine ligands to rhCCR2B mediates intracellular calcium release with similar rank-order potencies to those obtained in the binding experiments. MCP-1 elicited the strongest and most robust calcium flux while the other members of the MCP family led to slightly weaker calcium responses and had slightly lower potency than MCP-1. These relative functional potencies on the rhesus receptor are consistent with similar comparisons reported for the human receptor [3–5,34]. The potency of MCP-1 (rhesus, human, and murine) induced Ca^{2+} release was further confirmed in isolated rhesus PBMCs (data not shown). Although eotaxin competed with ^{125}I-rhMCP-1 and ^{125}I-hMCP-1 binding to rhCCR2, and this chemokine has been previously reported to be either an antagonist or a partial agonist for CCR2 [36,37], in our study, the affinity of eotaxin as a CCR2 ligand did not lead to an ability to functionally block the calcium flux elicited by the high affinity ligand MCP-1 (at 300 nM) nor was it a partial agonist in this assay at concentrations up to 100 nM (data not shown). It is therefore unclear if the affinity of eotaxin for CCR2 has any physiological relevance in rhesus monkeys.

Analysis of the blockade of ligand-mediated rhCCR2B activation by small molecule antagonists is a prerequisite for the use of these compounds in rhesus models of human disease. A small molecule that has been reported to be an antagonist for both human CCR2 and CCR5 receptors, TAK-779 [24], was tested for its affinity for the recombinant rhesus CCR2 receptor. In competition binding experiments, TAK-779 was very potent against rhesus CCR2 with a K_i of 0.5 nM (Fig. 7); we observed similar low nanomolar affinity for the hCCR2 expressed on human monocytes (data not shown). In addition, the binding of TAK-779 to rhCCR2 lead to functional receptor blockade as demonstrated by the dose-dependent inhibition of MCP-1-mediated calcium flux in the rhCCR2 recombinant CHO cell line with an IC_{50} of 0.8 nM.

In humans greater than 80% of monocytes express CCR2 [2]. After demonstrating that the commercially available antibody against human CCR2 binds rhesus CCR2, we tested PBMCs from rhesus blood for the expression pattern of CCR2. We identified monocytes by the side and forward scatter properties as well as reactivity with CD14, a typical marker of human monocytes. Similar to the observations in humans, >75% of rhesus monocytes were CCR2 positive (Fig. 6B).

The results described in this report suggest that rhCCR2B not only exhibits similar ligand potencies between the rhesus and human systems but the receptor is also expressed on the majority of blood-borne monocytes in both species. Therefore, rhesus animal models may serve well both for the evaluation of the role of CCR2B in human disease and potential therapeutic benefit of small molecule antagonists.

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