The CC Chemokine I-309 Inhibits CCR8-dependent Infection by Diverse HIV-1 Strains*

Richard Horuk‡‡, Joseph Hessegesser‡, Yiqing Zhou‡, Daryl Faulds‡, Meredith Halks-Miller‡, Susan Harvey‡, Dennis Taub¶, Michel Samson†, Marc Parmentier†, Joseph Rucker¶¶, Benjamin J. Doranz**‡‡‡, Robert W. Doms**

From the ‡Departments of Immunology, Pharmacology, and Cell Biology, Berlex Biosciences, Richmond, California 94804, the Laboratory of Immunology, NIA, National Institutes of Health, Baltimore, Maryland 21224, the ¶IRIBHN, Université libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Bruxelles, Belgium, and the **Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Using a chemokine receptor model based on known receptor sequences, we identified several members of the seven transmembrane domain G-protein superfamily as potential chemokine receptors. The orphan receptor ChemR1, which has recently been shown to be a receptor for the CC chemokine I-309, scored very high in our model. We have confirmed that I-309, but not a number of other chemokines, can induce a transient Ca²⁺ flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562 responded chemotactically in a dose-responsive manner to this chemokine. Given the importance of chemokines and their receptors in inflammation, autoimmunity, and the pathogenesis of AIDS, the identification and characterization of these proteins will be important to initiate approaches for therapeutic intervention. We used computer-assisted modelling, based on the sequences of the existing chemokine receptors, to identify potential chemokine receptors from the known data base. Using this approach, we identified a recently described orphan seven transmembrane domain receptor that has been known by several names, including ChemR1, TER-1, GPR-CY6, and CKRL1 (20–22), as a prime candidate for a chemokine receptor. Recently ChemR1 has been shown to be a ligand for the CC chemokine I-309 and is now designated CCR8 (23, 24). We have confirmed that I-309 but not a number of other chemokines can induce a transient Ca²⁺ flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562-expressed message for CCR8 receptor amino-terminal peptide cross-reacted with U-87 MG cells stably expressing CCR8, THP-1 cells, HL-60 cells, and human monocytes, a target cell for HIV-1 infection in vivo.

The chemokines are a diverse group of proteins that play an important role in host defense (1). They are classified into two major groups, CC and CXC, based on the position of the first two of their four invariant cysteines (2). Given their role in host defense, it is no surprise that chemokines and their receptors have been subjected to intense attack by pathogenic organisms. Some viruses have been shown to express viral chemokines and/or chemokine receptors (3–6) presumably as decoy proteins to help subvert the host immune response. Recently, human immunodeficiency virus type-1 (HIV-1) has been shown to utilize chemokine receptors as coreceptors to infect cells. These findings provide new opportunities not only to study HIV-1 pathogenesis but also to develop new anti-viral strategies.

While most HIV-1 strains use CD4 as a primary receptor, a specific chemokine receptor is also required for the membrane fusion reaction subsequent to virus infection. Generally, macrophage-tropic HIV-1 strains utilize CCR5 in conjunction with CD4 (7–11), while the T-cell tropic strains that typically emerge late in the course of the disease utilize CXCR4 (12, 13). In addition, dual-tropic viruses can efficiently utilize both of these receptors (8, 9, 14). Several other chemokine receptors have also been shown to function as coreceptors for a subset of HIV-1 strains, including CCR3 and CCR2b (8, 9). The importance of chemokine receptors for HIV-1 pathogenesis in vivo is shown by the finding that approximately 1% of Caucasians are homozygous for a 32-base pair deletion in CCR5 that prevents its transport to the cell surface. These individuals are very highly resistant to virus infection (15–19).

Given the importance of chemokines and their receptors in inflammation, autoimmunity, and the pathogenesis of AIDS, the identification and characterization of these proteins will be important to initiate approaches for therapeutic intervention. We used computer-assisted modelling, based on the sequences of the existing chemokine receptors, to identify potential chemokine receptors from the known data base. Using this approach, we identified a recently described orphan seven transmembrane domain receptor that has been known by several names, including ChemR1, TER-1, GPR-CY6, and CKRL1 (20–22), as a prime candidate for a chemokine receptor. Recently ChemR1 has been shown to be a ligand for the CC chemokine I-309 and is now designated CCR8 (23, 24). We have confirmed that I-309 but not a number of other chemokines can induce a transient Ca²⁺ flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562-expressed message for CCR8 was able to bind radiolabeled I-309 and responded chemotactically in a dose-responsive manner to this chemokine. We show here that CCR8 is a coreceptor for HIV-1 and that I-309 potently inhibited both HIV-1 envelope-mediated cell-cell fusion and virus infection of cells expressing CD4 and CCR8. Furthermore, we show that antibodies generated against the CCR8 receptor amino-terminal peptide detected CCR8 receptors in U-87 MG cells stably expressing CCR8 in addition to THP-1 and HL-60 cell lines and in human primary monocytes.

MATERIALS AND METHODS

Materials—Unlabeled chemokines were from Peprotech (Rocky Hill, NJ) or from R&D Systems (Minneapolis, MN). 125I-labeled chemokines were obtained from NEN Life Science Products (Boston, MA). Polyclonal antisera to CCR8 was raised in New Zealand White rabbits by

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§ To whom correspondence should be addressed: Berlex Biosciences, Dept. of Immunology, 15049 San Pablo Ave., Richmond, CA 94804, Tel.: 510-669-4625; Fax: 510-669-4244; E-mail: Horuk@crl.com.

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The abbreviations used are: HIV, human immunodeficiency virus type-1; RT-PCR, reverse transcriptase polymerase chain reaction; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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subcutaneous and intramuscular injection with the corresponding ami-
no-terminal domain for CCR8 conjugated to KLH. Following primary
immunization and six challenges with peptide, CCR8 antisera from
several pooled bleeds was collected and purified over a peptide affinity
column. Purified antibody was analyzed by peptide ELISA with a titer of
300,000. Tissue culture media was from Life Technologies, Inc. (Grand
Island, NY).

Cloning of CCR8 cDNA—Normal human thymus RNA (CLON-
TECH) was used as a template for reverse transcription followed by
reverse transcriptase polymerase chain reaction (RT-PCR). Based on
published sequences, two oligonucleotides, Oligo I (GGAGTGAATTG-
GTTACTTGTG) and Oligo II (TTATGCTCATTGATGCTC), were
synthesized and used to derive an 1135-base pair RT-PCR product
containing the open reading frame. The RT-PCR product was
cloned into pT7Blue (Novagen) and confirmed by complete DNA se-
quence analysis. For stable transfectants, the RT-PCR product was
cloned into pcDNA3 (Stratagene) expression vector containing G418
selection marker.

Cell Lines and Human Monocytes—Human U87 MG cells expressing
CCR8 were maintained in Dulbecco’s modified Eagle’s medium contain-
ing 10% heat-inactivated fetal bovine serum with 300 μg/ml of G418. Human
erythroleukemic cell line K562, monocytic cell line THP-1, promy-
elyocytic cell line HL-60 clone 15, and the T-cell line Jurkats were
maintained in RPMI 1640 medium (Life Technologies, Inc.) containing
10% fetal bovine serum. Differentiated HL-60 clone 15 cells were gen-
erated by treating cells with 0.5 μM butyric acid (Sigma) and 10 ng/ml
IL-5 (R&D Systems) as described (23). The murine embryo fibroblast
cell line PA317T4 was maintained in Dulbecco’s modified Eagle’s me-
dium-10 with CD4 expression selected for by the addition of 0.6 μg/ml
G418. All cell lines were obtained from the American Type Culture
Collection. Human monocytes were purified from peripheral blood of
healthy donors. Human buffy coats were obtained from Peninsula Blood
Collection. Human monocytes were separated using Ficoll-Hypaque as described (25) and two rounds of adher-
ence to plastic in RPMI 1640 medium with 10% fetal bovine serum.

Indirect Antibody Labeling FACS method—Human U-57 MG cells
were transfected with the pcDNA3 plasmid that encodes CCR8 as
described above. Stable cells were selected with G418. Purified CCR8
cytoplasmic antisera was incubated with cells expressing the receptors
antibody and cells were diluted 1:750 in PBS without Ca2+/Mg2+; for
blocking experiments, antibody and amino-terminal CCR8 peptide were
incubated together for 30 min on ice before adding to the addition to primary
were placed on ice for 30–60 min. Cells were pelleted with the super-
natant removed, placed back in PBS, and washed twice. Cells were then
incubated with goat anti-rabbit-FITC-conjugated antibody (diluted 1:1000
in PBS) on ice for 30 min. Cells were pelleted and washed as above. Controls for indirect labeling were (a) no primary,
secondary-FITC only, (b) pre-immune serum with secondary, (c) non-
transfected cells, (d) specific blocking with amino-terminal pep-
tide, and (e) cross-reactivity with CXCRI, CXCR2, CCR1, and CCR5
stably expressing cell lines.

Immunohistochemistry—Cultured cells were grown on 8-well, plastic
(Thermanox™) chamber slides and stained as described previously
(26), with the exception of adding Triton X-100 to buffers to maintain
membrane integrity. Cells were viewed on a Zeiss Axioskop and photo-
graphed with an attached Fuji HC-2000, three-chip CCD digital cam-
era. Images were first edited in Adobe Photoshop 3.0 to enhance con-
trast and white balance and then printed on a Fuji Pro tresk 3000
digital printer.

Sequence Comparison Using Gibbs Sampling—We used the MACAW
(Multiple Alignment Construction and Analysis Workbench) computer
program running on a Macintosh platform to generate a chemokine
receptor model based on the nine primary amino acid sequences for
CCR1–5 and CXCR1–4 to detect blocks of similarity. Two blocks corre-
sponding to positions 70–80 (SYLLNLALDDLFLFLTLPFW) and 286–
308 (TEVIAYTHCVCPVNYAVFGIF) of CCR1 were found, and simi-
lar blocks were located at comparable sites in each of the chemokine
receptors. This model was then used to search the TREMBL data base to
find homologous chemokine receptors.

Transfection—QT6 and HEK293 cells were transiently transfected
with plasmids encoding the CCR8 receptor by the calcium phosphate
precipitation method (8). After overnight expression, cells were re-
moved from the plate with 1 ml EDTA, centrifuged, and resuspended
in PBS for binding and biologic studies.

Chemokine Binding Studies—For binding assays cells (5 × 106 cells/

FIG. 1. Identification of a profile of chemokine receptors using the
program MACAW. The sequences shown as block 1 and block 2 correspond to regions of MACAW-detected sequence similarity among
CXCR1-CXCR4 and CCR1-CCR7. The sequences of the blocks were used as query sequences for the BLAST algorithm against the TREMBL
data base. A repeatedly high scoring sequence was CCR8, which is shown aligned below CCR7.

ml) were incubated in PBS (0.2 nm) and varying concentrations of
unlabeled ligands at room temperature for 30 min. The incubation was
terminated by removing aliquots from the cell suspension and separat-
ing cells from buffer by centrifugation through a silicone/paraffin oil
mixture as described previously (27). Nonspecific binding was deter-
mined in the presence of 1 μM unlabeled ligand.

Ca2+ Flux assays—CCR8 was expressed in HEK293 cells by tran-
sient transfection. Cells were loaded with 2.5 μM Fura-2/AM (Molecular
Probes) for 1 h, allowed to equilibrate in PBS, resuspended in PBS with Ca2+/Mg2+ - and warmed at 37 °C for 10 min
before measurement of ligand response. Ca2+ mobilization was meas-
ured in an Amino-Bowman Luminescence Spectrometer in a
constant stirring cuvette in a volume of 1.5 ml. Thrombin activating
peptide-4 was kindly provided by Lawrence Brass (University of Penn-
sylvania) and was used at a final concentration of 27 pm.

Chemotaxis—K562 cell migration was examined using a 48-well
microchemotaxis assay as described previously (28). The results were
expressed as the number of migrating cells per three high power fields
(× S.E.).

Gene Reporter Fusion Assays—Cell-cell fusion was monitored by a
luciferase-based gene reporter assay (10, 29). PA317T4 cells that stably
express human CD4 were transfected with luciferase-T7 and the de-
sired coreceptor. T7 RNA polymerase and envelope proteins were intro-
duced into effector HeLa cells by recombinant vaccinia viruses (29). To
initiate fusion, target and effector cells were mixed in 24-well plates at
37 °C. To assess the ability of I-309 to inhibit cell-cell fusion, target cells
were incubated with I-309 for 30 min at 37 °C prior to mixing with effector
cells. After 8 h, cells were lysed and assayed for luciferase activity.

RESULTS AND DISCUSSION

Chemokine receptors belong to a superfamly of seven trans-
membrane domain proteins that signal through coupled het-
erotrimeric G proteins (30). At the latest count, well over 600
members of this superfamily have been identified and classi-
ﬁed into families (31). However, a number of cloned serpentine
receptors exist for which no endogenous ligands have been
identiﬁed. To determine whether we could assign any of the
orphan receptors in the TREMBL data base to the chemokine
receptor family, we looked for regions of local similarity (blocks)
based on the protein sequence or the receptor for CC and CXC
chemokines, CCR1 through CCR5 and CXCR1 through
CXCR4, using a Gibbs sampling strategy (MACAW) (32). We
located two signiﬁcant blocks corresponding to the second
and third transmembrane segments (Fig. 1). We then used the
program BLAST (33) to identify sequences matching the 21
amino acid block near the second transmembrane segment and the 22 amino acid block near the third transmembrane segment. In addition to the known chemokine receptors, the highest scoring sequences represented orphan receptors. The orphan receptor that scored the highest was a protein known as ChemR1 where 23/23 amino acids (285–307) matched the block (Fig. 1). Although BLAST queries using the entire amino acid sequence of a particular chemokine receptor sequence (e.g., CCR1) also identified orphan receptors, we noted that known chemokine receptors (e.g., CCR5) scored worse than members of other families (e.g., angiotensin receptors) using this approach, and therefore, we considered its predictive value to be low. ChemR1 was the highest scoring orphan receptor in our analysis. It was recently identified as a receptor for the CC chemokine I-309 (23, 24) and is now designated CCR8. To confirm the ligand specificity of CCR8, we tested the ability of a number of CXC and CC chemokines including RANTES, MIP-1α, MIP-1β, Eotaxin, IL-8, MGSα, IP10, MCP-1, MCP-3, and I-309 to elicit an increase in Ca2+ flux in HEK293 cells transiently expressing the CCR8 receptor. As expected, only I-309 gave a transient calcium response (Fig. 2A). The EC50 for calcium mobilization by I-309 was approximately 1 ng/ml (Fig. 2B).

I-309 is a CC chemokine that was originally identified by subtractive hybridization of a cDNA library from an IL-2-de-pendent T-cell line (25). Recently, I-309 has been shown to protect murine T cell lymphomas against dexamethasone-induced apoptosis (34). Beyond these few reports, little is known regarding the real physiological role of I-309. It was originally described as a factor secreted by activated T cells that was able to stimulate the migration of human monocytes (35). Therefore, we tested the ability of I-309 to induce chemotaxis in a number of cell lines. We found that K562, which we and others have shown expresses CCR8 (20), and several human neuroblastoma cell lines2 stimulated with I-309, demonstrated a significant dose-dependent chemotactic response in vitro (Fig. 3). These cell lines exhibited typical bell-shaped dose-response curves in their migration, and the optimal I-309 concentration was between 10–100 ng/ml. Based on the finding that K562 cells expressed transcripts for CCR8 and responded chemotactically to I-309, we tested the ability of these cells to bind radiolabeled I-309. Although K562 cells incubated with 125I-labeled I-309 specifically bound the radiolabeled chemokine, the total binding was low (4200 cpm/106 cells), and the nonspecific binding ranged from 50 to 60% of the total.

Recently, members of the chemokine receptor family have been shown to serve as coreceptors for HIV-1. Macrophage-

![Fig. 2. I-309 mobilizes intracellular Ca2+ in cells transiently expressing the CCR8 receptor.](image)

A

| 100 nM |
|-------|
| CCR8  |
| pcDNA3|
| I309  |

B

| 100 ng/ml |
| I309  |
| I309  |
| I309  |
| I309  |

D. Taub, unpublished data.
tropic strains of HIV-1 use mainly CCR5 (7–11), T-cell line
tropic strains of HIV-1 use CXCR4 (12), and some viruses can
use other receptors including CCR2b, CCR3, STRL33, and V28
(8, 9, 14, 36, 37). Thus, to target chemokine receptors thera-
peutically with small molecule antagonists, it will be important
to define the range of chemokine receptors that can be utilized
by HIV-1 as coreceptors for invasion. We wanted to determine
whether CCR8 could serve as a coreceptor for HIV-1 and
whether I-309 could inhibit this. Thus, we used a cell-cell
fusion assay in which HeLa cells expressing the desired HIV-1
envelope protein and T7 polymerase are mixed with target cells
expressing CD4, a coreceptor, and luciferase under control of
the T7 promoter (8). If cell-cell fusion occurs, luciferase is pro-
duced as a consequence of cytoplasmic mixing. To determine if
I-309 could inhibit HIV-1 envelope-mediated cell-cell fusion,
cells expressing the ADA (macrophage-tropic) or BK132 (T-
cell tropic) envelope proteins were mixed with cells expressing
CD4 and CCR8. As shown in Fig. 4A, the ADA and BK132
envelope proteins mediated fusion with cells expressing CD4 and
CCR8. Fusion was strongly inhibited by I-309 in a dose-
dependent manner, providing further evidence that I-309 is a
CCR8 ligand (Fig. 4A). I-309 did not block fusion when either
CCR5 or CXCR4 were used as coreceptors (data not shown).
To determine if I-309 could also inhibit virus infection, cells
expressing CD4 and CCR8 were incubated with I-309 prior to
infection with HIV-1 ADA, which uses both CCR5 and CCR8 as
coreceptors. As shown in Fig. 4B, I-309 strongly inhibited in-
fecion by HIV-1 when cells expressed CCR8 but not when
CCR5 was expressed. Thus, I-309 inhibits CCR8-dependent
envelope-mediated cell-cell fusion and virus infection.
Since we have shown that CCR8 is a coreceptor for HIV-1, it
will be important to establish the relevance of this receptor in
the pathogenesis of AIDS by (a) identifying cells that express
the receptor, (b) ascertaining if they are targets of the virus,
and (c) determining the regulation of the receptor in these
cell types. In addition, it will be interesting to examine cells and
tissues of AIDS patients to determine whether CCR8 plays a
real role in HIV-1 transmission. To answer these and other
important questions will require immunological approaches
using CCR8 receptor antibodies. Since antibodies to a number
of chemokine receptors have been successfully raised using
amino-terminal peptides as immunogens (26), we generated
antibodies to CCR8 using a similar approach. The purified
anti-CCR8 polyclonal antisera were used to stain transfectants
expressing CCR8 receptors. The indirect immunofluorescence
was analyzed by flow cytometry. As shown in Fig. 5, the poly-
valent CCR8 receptor antibodies bound to the native receptor
expressed by these transfectants. This binding was specific
since it was inhibited by the addition of the CCR8 amino-
terminal peptide, and pre-immune serum did not stain these
cells (Fig. 5A). The antibodies were specific for CCR8 since
the antibodies did not recognize CXCR1-, CXCR2-, CCR1-, or
CCR5-transfected cells or untransfected U87-MG parental cells
(data not shown). To further investigate the ability of our CCR8
antisera to recognize other cell types that may express the
receptor, we carried out FACS analysis of three human cell
lines, THP-1, HL-60, and Jurkat, and also examined human
monocytes. The human monocytic cell line THP-1 has been
reported to respond chemotactically to the murine homolog of
I-309, TCA3, (38) while I-309 has been shown to induce an
increase in intracellular Ca2+ mobilization in human HL-60
cells induced with IL-5 and butyric acid (23). Human Jurkat
cells were recently shown to be negative for CCR8 mRNA (21).
As expected, FACS analysis of these cell lines revealed CCR8
expression on THP-1 and HL-60 cells while Jurkat cells showed
no staining (Fig. 5B–E). The staining was specific since it was
blocked by the CCR8 amino-terminal peptide. Interestingly, we
find that unstimulated HL-60 cells cross-react with the CCR8
antibodies (Fig. 5D), consistent with this they also bind 125I-
Labeled I-309. Tiffany et al. (23) have shown that only the differentiated HL-60 cells respond to I-309, which may be due to up-regulation and expression of intracellular proteins that allow CCR8 to transduce signals upon binding of ligand. Since human monocytes have clearly been shown to respond to I-309 by chemotaxis and by induction of cell migration (35), we examined these cells with the CCR8 antibodies. As seen in Fig. 5F, monocytes stained strongly with the antibodies.

To further test for chemokine receptor expression, human U-87 MG cells stably expressing CCR8 were immunohistochemically stained using antibodies to the CCR8 receptor. The cells were plated in 8-well chamber slides and stained as described previously (26). As can be seen in Fig. 6A, the U-87 MG cells stained strongly with the CCR8 antisera. However, not all of the cells stained with the antisera, which is consistent with the partial shift observed by indirect FACS analysis (Fig. 5A). The specificity of the CCR8 receptor staining was established by demonstrating that peptides specific for the antibodies could appropriately block antibody staining (Fig. 6B) and that specific staining was not observed with irrelevant monoclonal and polyclonal antibodies (data not shown).

Our findings that CCR8 functions as a coreceptor for diverse HIV-1 strains makes this molecule potentially relevant for viral pathogenesis. The ability of I-309 to inhibit CCR8-dependent virus infection should make it possible to determine if HIV-1 strains utilize this chemokine receptor for infection of relevant target cells in vivo. Indeed, we have clearly shown that CCR8 is expressed in human monocytes as a major target cell of HIV-1. The availability of antibodies to immunologically detect and characterize CCR8 should also make it possible to determine the relevance of this coreceptor in HIV-1 transmission.

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