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

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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 Ca^{2+} flux in cells expressing CCR8. In addition, the human erythroleukemic cell line K562 responded chemotactically in a dose-responsive manner to this chemokine. Since several chemokine receptors have been shown to be required as coreceptors for HIV-1 infection, we asked whether human immunodeficiency virus type 1 (HIV-1) could efficiently utilize CCR8. Here we show that the CCR8 receptor can serve as a coreceptor for diverse T-cell tropic, dual-tropic, and macrophage-tropic HIV-1 strains and that I-309 was a potent inhibitor of HIV-1 envelope-mediated cell-cell fusion and virus infection. Furthermore, we show by flow cytometry and immunohistochemistry that antibodies generated against the 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 infectivity *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)[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 Ca^{2+} 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). ^{125}I-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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[1] 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 amino-terminal domain for CCR8 conjugated to KLH. Following primary immunization and six challenges with peptide, CCR8 antiserum from several pooled bleeds was collected and purified over a peptide affinity column. Purified antibody was analyzed by peptide ELISA with a titer of 30,000. Tissue culture media was from Life Technologies, Inc. (Grand Island, NY).

Cloning of CCR8 cDNA—Normal human thymus RNA (CLONTECH) 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 (GGAGTGAATGTGTTTTATGTG) and Oligo II (ATTTAGTCTTCATTGATCCTCAC), were synthesized and used to derive an 1135-base pair RT-PCR product corresponding to the open reading frame. The RT-PCR product was cloned into pT7Blue (Novagen) and confirmed by complete DNA sequence 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 containing 10% heat-inactivated fetal bovine serum with 300 μg/ml of G418. Human embryonic kidney (HEK) 293 cells and the Japanese quail fibrosarcoma line QT6-C5 were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated bovine serum. The human erythroleukemic cell line K562, monocytic cell line THP-1, promyelocytic cell line HL-60 clone 15, and the T-cell line Jurkat were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum. Differentiated HL-60 clone 15 cells were generated 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 medium-10 with CD4 expression selected for by the addition of 0.6 mg/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 Bank (Burlingame, CA), and peripheral blood monocytes were separated using Ficoll-Hypaque as described (25) and two rounds of adherence to plastic in RPMI 1640 medium with 10% fetal bovine serum.

Indirect Antibody Labeling FACS method—Human U-87 MG cells were transfected with the pcDNA3 plasmid that encodes CCR8 as described above. Stable cells were selected with G418. Purified CCR8 polyclonal antisera was incubated with cells expressing the receptors (antibody and cells were diluted 1:750 in PBS without Ca^{2+}/Mg^{2+}; for blocking experiments, antibody and amino-terminal CCR8 peptide were incubated together for 30 min on ice prior to addition to cells). Cells were placed on ice for 30–60 min. Cells were pelleted with the supernatant 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 in the dark 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 cell staining, (*d*) specific blocking with amino-terminal peptide, and (*e*) cross-reactivity with CXCR1, 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 photographed with an attached Fuji HC-2000, three-chip CCD digital camera. Images were first edited in Adobe Photoshop 3.0 to enhance contrast and white balance and then printed on a Fuji Pictrography 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 corresponding to positions 70–80 (SIYLLNLAISDLLFLFTLPFW) and 286–308 (TEVIAYTHCCVNPVIYAFVGERF) of CCR1 were found, and similar 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 potential 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 removed from the plate with 1 mM EDTA, centrifuged, and resuspended in PBS for binding and biologic studies.

Chemokine Binding Studies—For binding assays cells (5×10^5 cells/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 separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (27). Nonspecific binding was determined in the presence of 1 μM unlabeled ligand.

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CXCR1   DTFLLHLAVADTLLVLTLPLW     YAGALLLACISFDRYLNIVHAT
CXCR2   DVYLLNLALADLLFALTLPIW     YSGILLLACISVDRYLAIVHAT
CXCR3   DVYLLNLALADLLFALTLPIW     YSGILLLACISVDRYLAIVHAT
CXCR4   DKYRLHLSVADLLFVITLPFW     YSSVLILAFISLDRYLAIVHAT
CCR1    SIYLLNLAISDLLFLFTLPFW     YSEIFFIILLTIDRYLAIVHAV
CCR2    DIYLLNLAISDLLFLITLPLW     FGGIFFIILLTIDRYLAIVHAV
CCR3    NIYLLNLAISDLLFLVTLPFW     YSEIFFIILLTIDRYLAIVHAV
CCR4    DVYLLNLAISDLLFVFSLPFW     YSGIFFVMLMSIDRYLAIVHAV
CCR5    DIYLLNLAISDLFFLLTVPFW     FGGIFFIILLTIDRYLAVVHAV
CCR6    DVYLVNMAIADILFVLTLPFW     NCGMLLLTCISMDRYIAIVQAT
CCR7    DTYLLNLAVADILFLLTLPFW     FSGMLLLLCISIDRYVAIVQAV

CCR8    DVYLLNLALSDLLFVFSFPFQ     YSSMFFITLMSVDRYLAVVHAV
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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.

Ca^{2+} Flux; assays—CCR8 was expressed in HEK293 cells by transient transfection. Cells were loaded with 2.5 μM Fura-2/AM (Molecular Probes) at 37 °C in the dark for 1 h, allowed to efflux for 15 min in PBS, resuspended in PBS with Ca^{2+}/Mg^{2+}, and warmed at 37 °C for 10 min before measurement of ligand response. Ca^{2+} mobilization was measured in an Aminco-Bowman Luminescence Spectrometer in a constantly stirring cuvette in a volume of 1.5 ml. Thrombin activating peptide-4 was kindly provided by Lawrence Brass (University of Pennsylvania) and was used at a final concentration of 27 μM.

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 desired coreceptor. T7 RNA polymerase and envelope proteins were introduced 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.

Infection Studies—Viral stocks were prepared by transfecting HEK293 cells with plasmids encoding the desired envelope and the NL4–3 luciferase virus backbone (pNL-Luc-E-R-) (10). For infection, U87-MG cells were plated in 24-well plates and transfected with pT4 and the desired coreceptor. The medium was changed after 6 h, and cells were allowed to express overnight. Cells were infected the next day with 500 μl of viral supernatant. Media was changed the following day, and 0.5 ml of additional media was added 1 day prior to harvest of cells. Cells were lysed at 4 days post-infection by resuspension in 150 μl of 0.5% Nonidet P-40/PBS, and 50 μl of the resulting lysate was assayed for luciferase activity.

RESULTS AND DISCUSSION

Chemokine receptors belong to a superfamily of seven transmembrane domain proteins that signal through coupled heterotrimeric G proteins (30). At the latest count, well over 600 members of this superfamily have been identified and classified 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 sequences of the receptors 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

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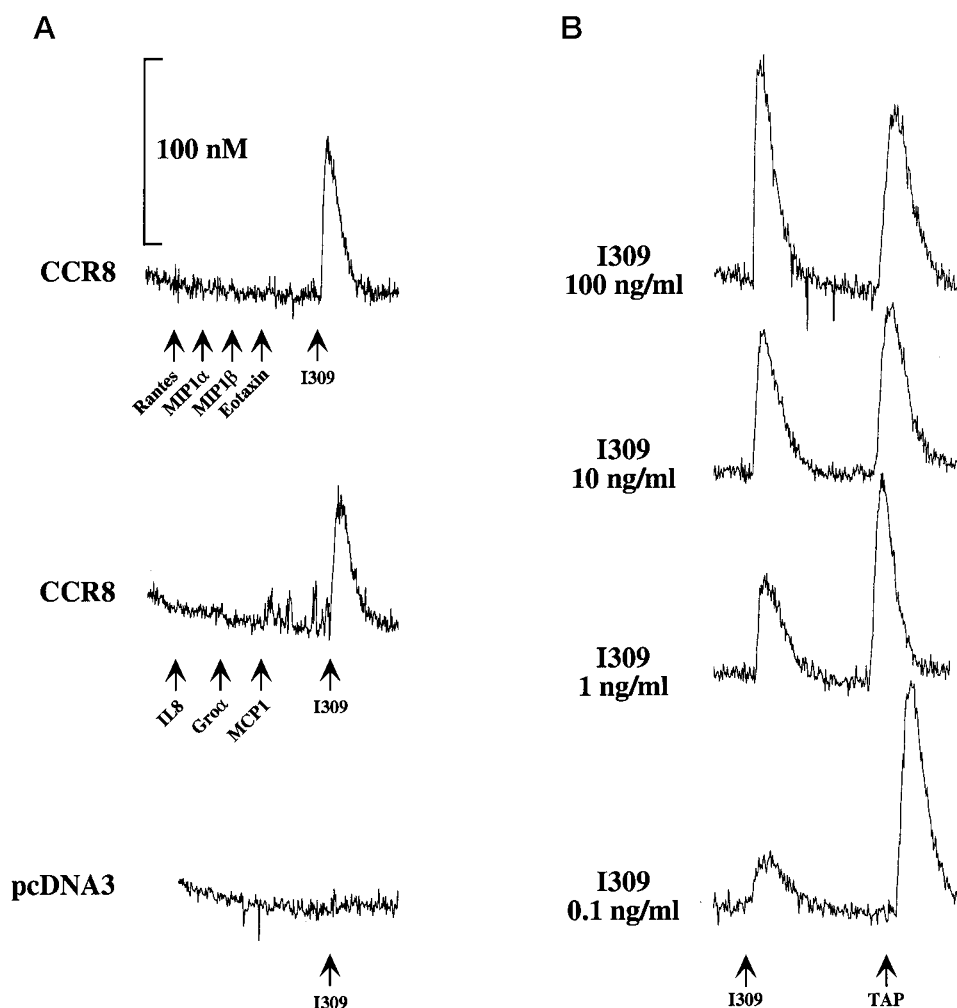

FIG. 2. **I-309 mobilizes intracellular Ca^{2+} in cells transiently expressing the CCR8 receptor.** *A*, HEK293 cells transfected with pcDNA3-CCR8 or the control plasmid pcDNA3 were loaded with Fura-2 and stimulated with 100 nM of the indicated chemokines. *B*, HEK293 cells transfected with pcDNA3-CCR8 were loaded with Fura-2 and stimulated with increasing concentrations of I-309. Dilutions below 0.1 ng/ml I-309 did not yield signals above background. Activation of the thrombin receptor by thrombin-activating peptide-4 (TAP), in both *panel A* (data not shown) and *panel B*, indicated that cells were fully capable of signaling.

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, MGSA, IP10, MCP-1, MCP-3, and I-309 to elicit an increase in Ca^{2+} flux in HEK293 cells transiently expressing the CCR8 receptor. As expected, only I-309 gave a transient calcium response (Fig. 2*A*). The EC_{50} for calcium mobilization by I-309 was approximately 1 ng/ml (Fig. 2*B*).

I-309 is a CC chemokine that was originally identified by subtractive hybridization of a cDNA library from an IL-2-dependent 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 lines[2] 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 ^{125}I-labeled I-309 specifically bound the radiolabeled chemokine, the total binding was low (4200 cpm/10^6 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-

[2] D. Taub, unpublished data.

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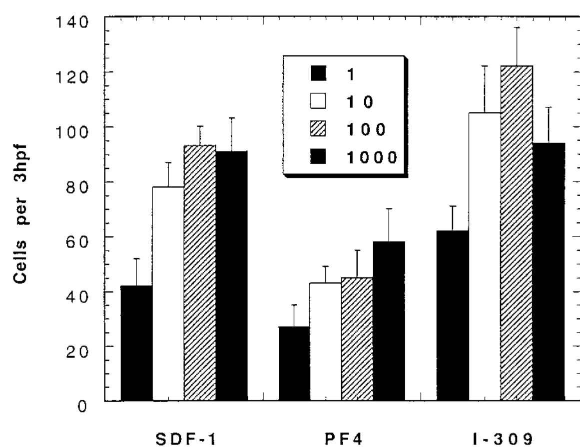

FIG. 3. **I-309 induces cell migration in K562 cells.** Cells were tested for their ability to migrate in response to various concentrations of the CXC chemokines IL-8 and PF4 and the CC chemokine I-309, as described under "Materials and Methods." The results are expressed as the number of cells per three high power fields (*3hpf*) ± S.E.

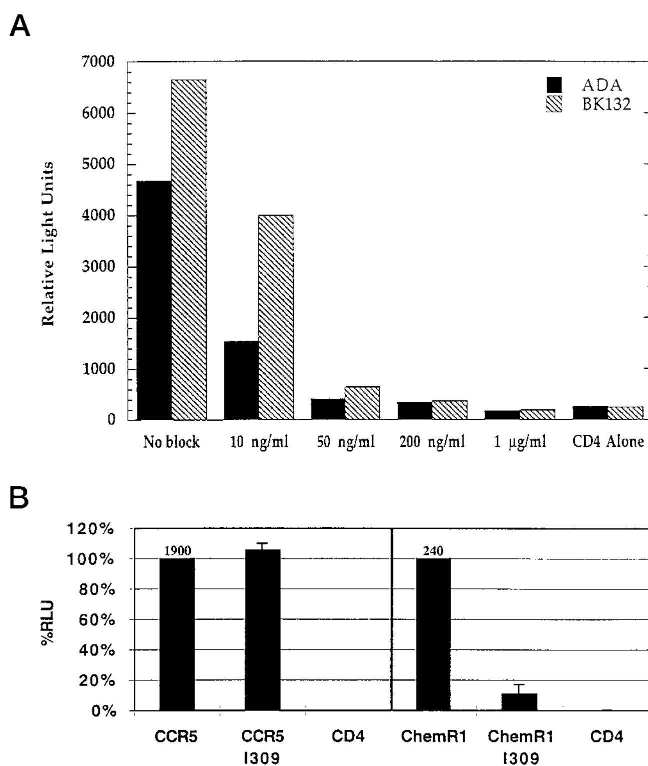

FIG. 4. **CCR8-dependent cell-cell fusion and virus infection are inhibited by I-309.** *A*, cell-cell fusion was determined by mixing murine PA317T4 cells (which express human CD4) expressing CCR8 and containing the luciferase gene under the control of the T7 promoter with HeLa cells expressing T7 polymerase and either ADA (macropohage-tropic) or BK132 (T-cell tropic) envelope protein. I-309, at the indicated concentrations, was incubated with target cells for 30 min at 37 °C prior to mixing with effector cells. The degree of cell-cell fusion was determined by measuring relative light units 8 h after mixing. I-309 showed no inhibitory effect against CCR5- or CXCR4-dependent cell-cell fusion at 1 μg/ml (data not shown). *B*, U87-MG CCR8 cells were plated in 24-wells and transfected with pT4 (encoding CD4) and the desired coreceptor. I-309 was added at a concentration of 100 ng/ml 30 min prior to infection with 500 μl of viral supernatant containing a luciferase-reporter virus bearing the ADA envelope protein. Cells were lysed at 4 days post-infection, and the lysate was assayed for luciferase activity. *RLU*, relative light units.

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 therapeutically 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 produced as a consequence of cytoplasmic mixing. To determine if I-309 could inhibit HIV-1 envelope-mediated cell-cell fusion, cells expressing the ADA (macropohage-tropic) or BK132 (T-cell tropic) envelope proteins were mixed with cells expressing CD4 and CCR8. As shown in Fig. 4*A*, 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. 4*A*). 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. 4*B*, I-309 strongly inhibited infection 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 polyvalent antisera were used to stain transfectants expressing CCR8 receptors. The indirect immunofluorescence was analyzed by flow cytometry. As shown in Fig. 5, the polyvalent 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. 5*A*). 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 Ca^{2+} 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. 5, *B-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. 5*D*), consistent with this they also bind ^{125}I-

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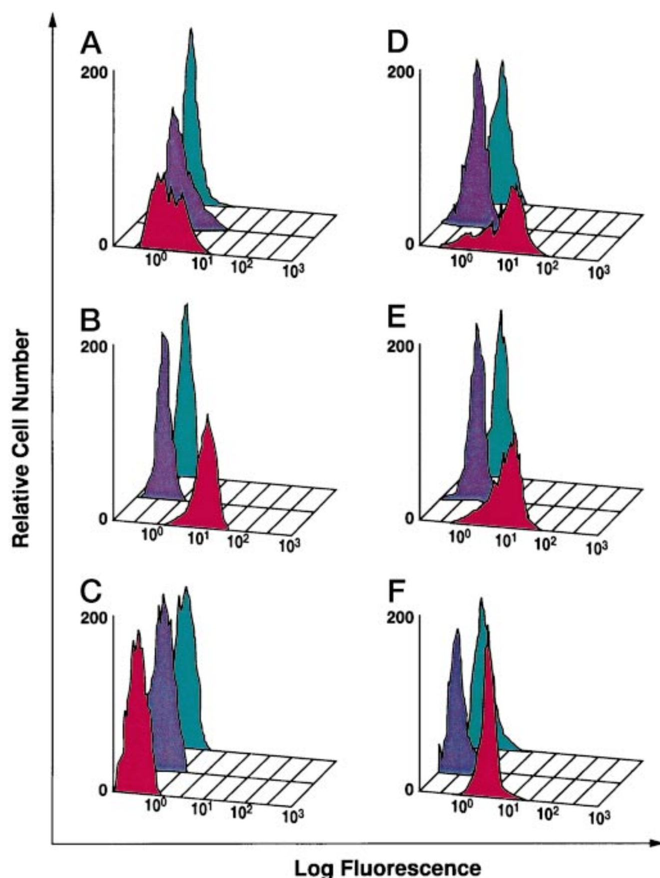

FIG. 5. **Antibodies to CCR8 amino-terminal peptides recognize the CCR8 receptor by flow cytometric analysis.** Several cell lines were incubated with CCR8 receptor antibodies in the presence and absence of CCR8 amino-terminal peptide and examined by flow cytometry. U87 MG cells stably expressing the CCR8 receptor (*A*), THP-1 (*B*), Jurkat (*C*), HL-60 clone 15 cells (*D*), HL-60 clone 15 cells treated with IL-5 and butyric acid (*E*), and primary human monocytes (*F*). *Red* shifts represent incubation of cells with anti-CCR8 antibodies and goat anti-rabbit-IgG FITC secondary antibody. *Green* shifts represent incubation of cells with pre-immune sera and goat anti-rabbit-IgG FITC secondary antibody. *Purple* shifts represent incubation of cells with anti-CCR8 antibodies in the presence of CCR8 amino-terminal peptide followed by incubation with goat anti-rabbit-IgG FITC secondary antibody.

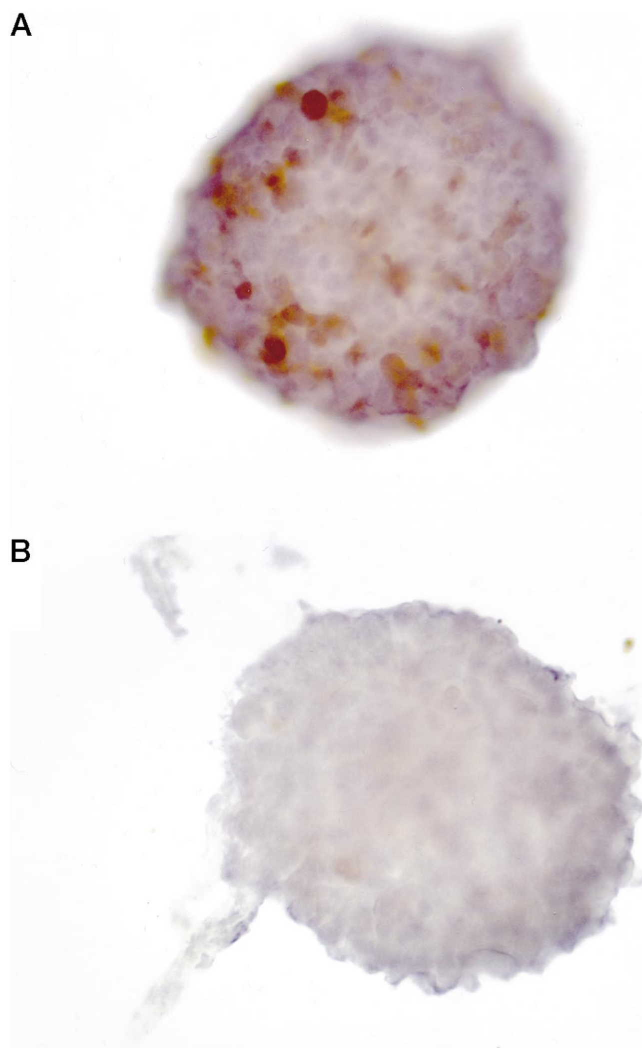

FIG. 6. **Antibodies to CCR8 amino-terminal peptides immunohistochemical stain cells expressing the CCR8 receptor.** Human U-87 MG glioblastoma cells transfected with CCR8 receptors were stained with antibodies to CCR8 in the absence (*A*) and presence (*B*) of peptides specific for the antibody.

labeled I-309.[3] 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. 5*F*, 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. 6*A*, 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. 5*A*). The specificity of the CCR8 receptor staining was established by demonstrating that peptides specific for the antibodies could appropriately block antibody staining (Fig. 6*B*) and that specific staining was not observed with irrelevant monoclonal and polyclonal antibodies (data not shown).

[3] J. Hesselgesser, unpublished data.

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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