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Role of CCR5 in Infection of Primary Macrophages and Lymphocytes by Macrophage-Tropic Strains of Human Immunodeficiency Virus: Resistance to Patient-Derived and Prototype Isolates Resulting from the Δ*ccr5* Mutation

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The α-chemokine receptor fusin (CXCR-4) and β-chemokine receptor CCR5 serve as entry cofactors for T-cell (T)-tropic and macrophage (M)-tropic human immunodeficiency virus type 1 (HIV-1) strains, respectively, when expressed with CD4 in otherwise nonpermissive cells. Some M-tropic and dual-tropic strains can also utilize other β-chemokine receptors, such as CCR2b and CCR3. A mutation of CCR5 (Δ*ccr5*) was recently found to be common in certain populations and appears to confer protection against HIV-1 in vivo. Here, we show that this mutation results in a protein that is expressed intracellularly but not on the cell surface. Primary CD4 T cells from Δ*ccr5* homozygous individuals were highly resistant to infection with prototype M-tropic HIV-1 strains, including an isolate (YU-2) that uses CCR5 and CCR3, but were permissive for both a T-tropic strain (3B) and a dual-tropic variant (89.6) that uses CXCR-4, CCR5, CCR3, or CCR2b. These cells were also resistant to M-tropic patient isolates but were readily infected by T-tropic patient isolates. Primary macrophages from Δ*ccr5* homozygous individuals were also resistant to infection with M-tropic strains, including YU-2, but the dual-tropic strain 89.6 was able to replicate in them even though macrophages are highly resistant to CXCR-4-dependent T-tropic isolates. These data show that CCR5 is the essential cofactor for infection of both primary macrophages and T lymphocytes by most M-tropic strains of HIV-1. They also suggest that CCR3 does not function for HIV-1 entry in primary lymphocytes or macrophages, but that a molecule(s) other than CCR5 can support entry into macrophages by certain virus isolates. These studies further define the cellular basis for the resistance to HIV-1 infection of individuals lacking functional CCR5.

T4 lymphocytes and macrophages are the two major targets for productive human immunodeficiency virus type 1 (HIV-1) infection in vivo. Strains can be broadly grouped into macrophage (M)-tropic isolates, which infect primary macrophages and lymphocytes but not transformed cell lines, and T-cell (T)-tropic isolates, which infect CD4-positive transformed cells and primary lymphocytes but not macrophages. While the T-tropic strains frequently form syncytia in infected lymphocytes (syncytium-inducing [SI] strains), M-tropic strains typically do not (non-syncytium-inducing [NSI] strains). Isolates obtained from newly infected individuals are nearly always M tropic, indicating that these variants are important for person-to-person spread (42, 43). They are also the predominant species found during the long asymptomatic phase, suggesting a role in viral persistence (22, 35, 39). In contrast, T-tropic strains emerge later in about 50% of infected individuals, concurrent with disease progression, and, although AIDS can develop in the absence of T-tropic variants, their emergence is associated with accelerated immune decline (21, 22, 39). The viral and cellular basis for differential host tropism has been the focus of intense interest, largely because of this relationship between viral phenotypes and aspects of pathogenesis such as transmission and disease progression.

HIV-1 requires cofactor molecules in addition to CD4 for fusion and entry into target cells, and the cofactors utilized by particular strains are a major determinant of host cell tropism. Otherwise nonpermissive CD4-positive cell lines can be made permissive for entry of T-tropic strains by the seven-transmembrane α-chemokine (CXC) receptor fusin (CXCR-4) (16). In contrast, permissiveness for M-tropic strains is conferred by the β-chemokine (CC) receptor CCR5 (2, 5, 13–15). Other chemokine receptors can also serve as entry cofactors for certain virus strains, including CCR3 and CCR2b (5, 14). Since these cofactors have been identified largely in systems in which specific molecules are introduced into cell lines that otherwise do not support HIV fusion and infection, they show that a particular molecule is sufficient for HIV entry but leave open the question of whether it is necessary, or the only cofactor, for infection of normal host cells. Some HIV-1 strains can use a broad spectrum of cofactors (14), and it is quite possible that other not yet identified molecules can also function to facilitate

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HIV entry. Defining the roles played by these and potentially other cofactors in infection of HIV's natural targets is of great importance both for the understanding of HIV pathogenesis and for the development of therapeutic agents designed to target cofactor-mediated HIV entry.

Recently, a mutation of CCR5 (Δ*ccr5*) that results in premature truncation of the molecule was identified. This mutation was common among Caucasian populations but was present at significantly reduced frequency among comparable HIV-1-infected people, and individuals homozygous for the Δ*ccr5* allele were completely absent from the HIV-1-infected groups (12, 32). Similarly, two individuals in a group of people who remained uninfected despite repeated exposure were found to be homozygous for the same Δ*ccr5* mutation, and these individuals' lymphocytes were resistant in vitro to M-tropic strains of HIV-1 but permissive for T-tropic strains (24, 29). This indicates that the Δ*ccr5* mutation confers resistance to HIV-1 infection in vivo and is consistent with the important role of M-tropic viruses in transmission.

To better understand the roles played by CCR5 and other cofactors relevant to HIV infection in vivo, it is necessary to define their functions in infection of primary target cells and test their importance for both prototype and patient-derived viral isolates. We utilized primary lymphocytes and macrophages from individuals carrying the Δ*ccr5* allele as a naturally occurring knockout model and examined the replication of several HIV-1 isolates with different characteristics. We found that macrophages and lymphocytes homozygous for Δ*ccr5* were highly resistant to prototype viruses and patient isolates with typical M-tropic/NSI characteristics, including a strain that can utilize both CCR5 and CCR3. In contrast, neither primary nor prototype T-tropic/SI isolates were restricted in these lymphocytes, and a promiscuous HIV-1 strain that can use CXCR-4, CCR5, CCR3, or CCR2b replicated in both macrophages and lymphocytes. Our results show that CCR5 is the principal cofactor utilized for infection of both macrophages and lymphocytes by M-tropic strains, suggest that CCR3 is unlikely to play an important role in HIV-1 entry into its major target cells, and indicate that other pathways in addition to CCR5 may be utilized by particular isolates for entry into primary macrophages.

MATERIALS AND METHODS

Primary blood cells. Healthy volunteers were screened for the Δ*ccr5* mutation by PCR amplification of genomic DNA as previously described (32). Peripheral blood mononuclear cells (PBMC) were obtained by the Ficoll-Hypaque method from heparinized whole blood. Peripheral blood lymphocytes (PBL) were derived from PBMC by serial depletion of adherent cells and were maintained in RPMI supplemented with 20% fetal bovine serum (FBS), glutamine (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). PBL were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 3 to 4 days prior to infection and were maintained thereafter with interleukin 2 (IL-2; 20 U/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Depletion of CD8 cells was carried out with immunomagnetic beads (Dynal Corp., Long Island, N.Y.) following the instructions of the manufacturer. Monocyte-derived macrophages (MDM) were isolated from PBMC by a two-step adherence procedure as described elsewhere (8), plated at 4×10^5 cells per well in 24-well plates, and maintained for 7 to 10 days prior to infection to allow differentiation into macrophages. Cells were maintained in Dulbecco modified Eagle medium containing 10% FBS, 10% horse serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were supplemented with macrophage colony-stimulating factor (100 U/ml; Genetics Institute, Cambridge, Mass.). In specified experiments, MDM were isolated from PBMC by plastic adherence (4), followed by multiple rounds of vigorous washing.

Infections. PHA-stimulated PBL (4×10^5 cells) were infected overnight with equal amounts of each virus, washed twice, and plated in 200 μl of medium containing IL-2 in round-bottom microtiter tissue culture plates. Twice weekly, 50% of the medium was removed for p24 antigen determination and replaced with fresh medium. MDM were allowed to differentiate for 7 to 10 days prior to infection, infected overnight with equal amounts of virus, washed three times, and refed. Supernatant was sampled twice weekly and replaced with fresh medium. Supernatant viral p24 antigen was measured by enzyme-linked immunosorbent assay (ELISA; Dupont, Wilmington, Del.). To detect proviral DNA formation, cells were infected as described above with DNase-treated virus stocks, lysed 3 days later, and subjected to 35 cycles of PCR amplification with a primer set (long terminal repeat [LTR] plus/LTR minus) that amplifies a 430-bp region of U3/U5 LTR sequences. PCR products were resolved by agarose gel electrophoresis, transferred to Nylon membranes, probed with a ^{32}P-end-labeled oligonucleotide probe, and analyzed by autoradiography. The primers and probe and the details of this procedure have been described previously (14). Virus stocks were grown in CEMX174 cells (89.6 and 3B), PM-1 cells (JR-FL and BAL), or PBL (SF162, YU-2, and primary isolates), and inocula were equalized on the basis of p24 antigen content. All infections were carried out in a blinded manner on coded samples.

Generation of polyclonal antibody for CCR5 detection. A region encoding the first 35 amino-terminal residues of CCR5, which lies within the first extracellular domain of the protein, was cloned into pGEX-2T (Pharmacia, Piscataway, N.J.) and expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferease (GST). Protein was purified by glutathione-Sepharose affinity chromatography and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and rabbits were immunized four times with purified protein. Immune rabbit serum was then absorbed with GST-glutathione-Sepharose to remove antibodies to GST. To confirm the specificity of the antibody, Western blotting (immunoblotting) was carried out against GST, CCR5-GST, and the CCR5 peptide released from the fusion protein by thrombin cleavage and by flow cytometry of HOS4 cells that were stably transfected with CCR5 or with control vector alone.

Immunofluorescence detection of CCR5. QT6 quail cells were plated on glass coverslips and transfected with 5 μg of pCDNA3 (Invitrogen, San Diego, Calif.) expression plasmids containing *CCR5* or Δ*ccr5* or vector alone by the calcium phosphate method. Expression plasmids and transfections have been described previously (14, 32). Forty-eight hours after transfection, cells were stained for *CCR5* expression by using a rabbit polyclonal antiserum raised against the N-terminal region of CCR5. To detect both intracellular and extracellular antigen, the cells were fixed and permeabilized (0.1 M sodium cacodylate [pH 7.3], 50 mM potassium ferricyanide, 1 mM calcium chloride, 4% paraformaldehyde, 0.15% Triton X-100) for 1 h at room temperature prior to the addition of the primary antibody. To detect surface expression only, the cells were fixed and permeabilized as described above following the primary antibody steps. Staining was carried out for 1 h at 4°C with rabbit antiserum diluted 1:500 in phosphate-buffered saline (PBS) containing 5% FBS and 10 μg of ethidium bromide (included to stain nuclei of cells that were permeable to the primary antibody) per ml. After washing, they were then incubated for 1 h at 4°C with fluorescein isothiocyanate-conjugated donkey anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, West Grove, Pa.) diluted 1:100 in PBS containing 4,6 diamidino-2-phenylindole (DAPI; 500 ng/ml; Sigma Chemical Co., St. Louis, Mo.) as a nuclear stain. The coverslips were then washed, mounted, and examined by immunofluorescence microscopy with a digital imaging system. Red, blue, and green channels were digitized separately and then recompiled for analysis (Photoshop; Adobe Systems, Mountain View, Calif.).

RESULTS

The Δ*ccr5* mutation results in intracellular but not surface protein expression. We have shown that a mutant allele of the *CCR5* gene, termed Δ*ccr5*, is common in European Caucasians but is present at a reduced frequency among comparable HIV-1-infected groups (32). Most strikingly, no homozygous individuals were found among HIV-1-infected persons in two separate studies (12, 32). This allele possesses a 32-bp deletion which results in premature truncation of the predicted protein within the third extracellular domain. While expression of wild-type *CCR5* in conjunction with CD4 renders nonhuman cell types susceptible to *env*-mediated fusion and infection by M-tropic strains of HIV-1, expression of the Δ*ccr5* gene does not (32). This mutant allele has also been identified in several individuals who were repeatedly exposed to HIV but remained uninfected (24).

In order to detect CCR5, a polyvalent antibody was raised against the N-terminal domain of CCR5 expressed as a GST fusion protein, which is within a region of the molecule predicted to be present on both the wild-type and mutant proteins. The absorbed antibody is specific for CCR5 as determined by Western blotting against GST, CCR5-GST, and the CCR5 peptide released from the fusion protein by thrombin cleavage (data not shown). In addition, flow cytometry of transfected cells showed that immune serum, but not preimmune serum,

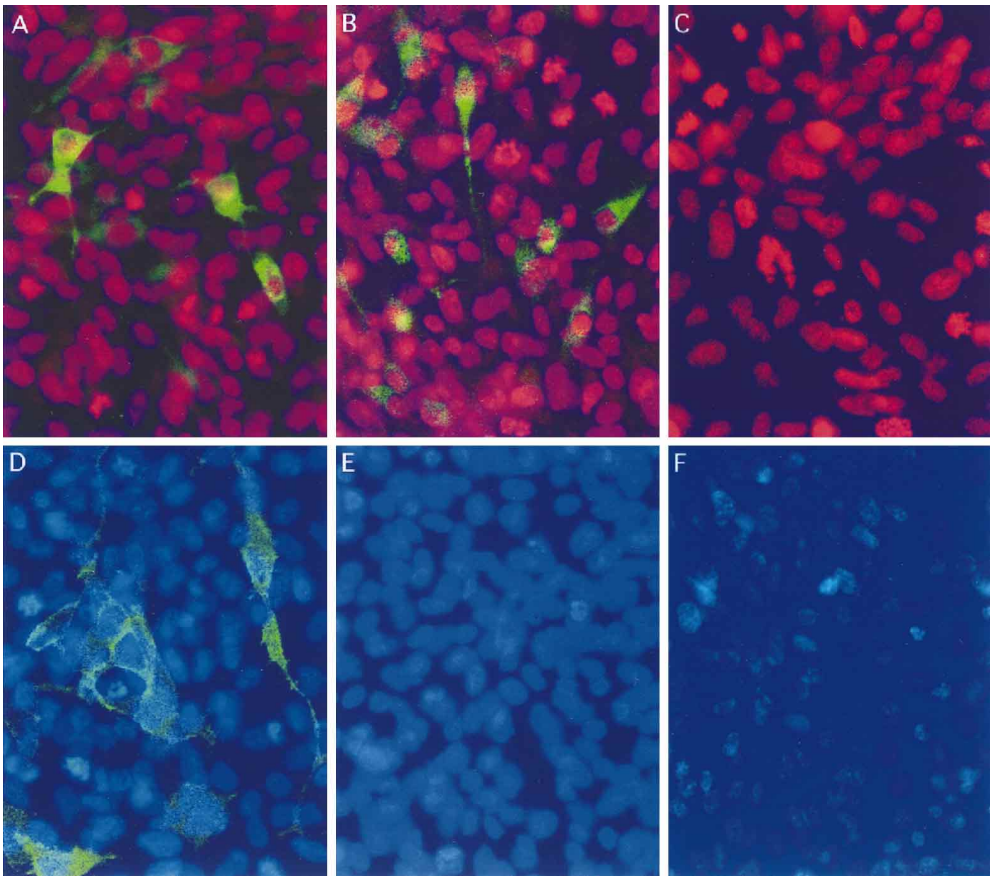

FIG. 1. Expression of wild-type and mutant CCR5. QT6 quail cells were transfected with expression plasmids for the wild-type *CCR5* (A and D) or the Δ*ccr5* mutant (B and E) or with control plasmid (C and F) and were stained by indirect immunofluorescence as described in Materials and Methods. Cells were either permeabilized prior to addition of primary antibody to detect both intracellular and extracellular expression (A to C) or were left intact until after primary antibody staining so that only extracellular expression would be detected (D to F). Ethidium bromide (red) was added to the primary antibody and DAPI (blue) was added to the secondary antibody so that red nuclear staining in prefixed cultures (A to C) would show that these cells were permeable to the primary antibody, while the absence of red staining in postfixed cells (D to F) would confirm that only extracellular expression would be detected. Wild-type CCR5 was detected in both permeabilized (A) and intact (D) cells, indicating surface expression, while Δccr5 was detected only when cells were permeabilized (B) and not in intact cells (E), indicating that the mutant protein was not expressed on the cell surface.

resulted in specific staining of *CCR5*-transfected but not control cells, and this staining was inhibited by the CCR5 N-terminal peptide but not by control (GST) peptide (data not shown). To define the pattern of expression directed by the mutant Δ*ccr5* gene, quail QT6 cells were then transfected with wild-type or Δ*ccr5* plasmids and stained by indirect immunofluorescence (Fig. 1). To distinguish intracellular from surface expression, cells were either fixed and permeabilized prior to the addition of primary antibody to allow staining of both intracellular and surface protein (Fig. 1A to C) or were fixed after the primary antibody stain (Fig. 1D to F) so that only surface proteins would be detected. Ethidium bromide was included along with the primary antibody and DAPI was included with the secondary antibody so that nuclear staining with ethidium bromide (red) would confirm that cells were permeable at the time of primary detection, while DAPI (blue) nuclear staining confirmed that intracellular antigen was not accessible to primary antibody and only surface expression would be detected.

Transfection of wild-type *CCR5* resulted in staining of nonpermeabilized (Fig. 1D) as well as permeabilized cells (Fig. 1A), indicating that protein was expressed and transported to the cell surface. In contrast, surface expression was not detected after Δ*ccr5* transfection in cells that were maintained intact (Fig. 1E) but was seen when cells were first made permeable to the antibody (Fig. 1B). This indicates that the protein encoded by Δ*ccr5* is expressed but fails to reach the cell surface like the wild type. Efforts to detect endogenous CCR5 on primary human cells or human cell lines have so far not been successful, likely due to levels of protein lower than those with transfected cells.

Resistance of Δ*ccr5* PBL to infection by M-tropic but not T-tropic or dual-tropic strains. To investigate the cellular basis for the resistance to HIV infection resulting from the Δ*ccr5* mutation, to better understand the role of CCR5 in infection of primary cell types, and to compare CCR5 dependence of different viral strains, we infected blood leukocytes derived from individuals carrying the Δ*ccr5* mutation. Donors were screened by PCR amplification of genomic *CCR5* sequences followed by restriction digestion, which distinguishes the longer wild-type allele from the smaller deleted sequence (32). Since these individuals were identified by systematic screening and were not targeted because of suspected or demonstrated resistance to HIV infection, they represent an unselected group of individuals carrying the Δ*ccr5* allele. Genotypically normal individuals from the same population served as controls. PBL from donors possessing differing complements of *CCR5* alleles were infected with several well-characterized prototype HIV-1

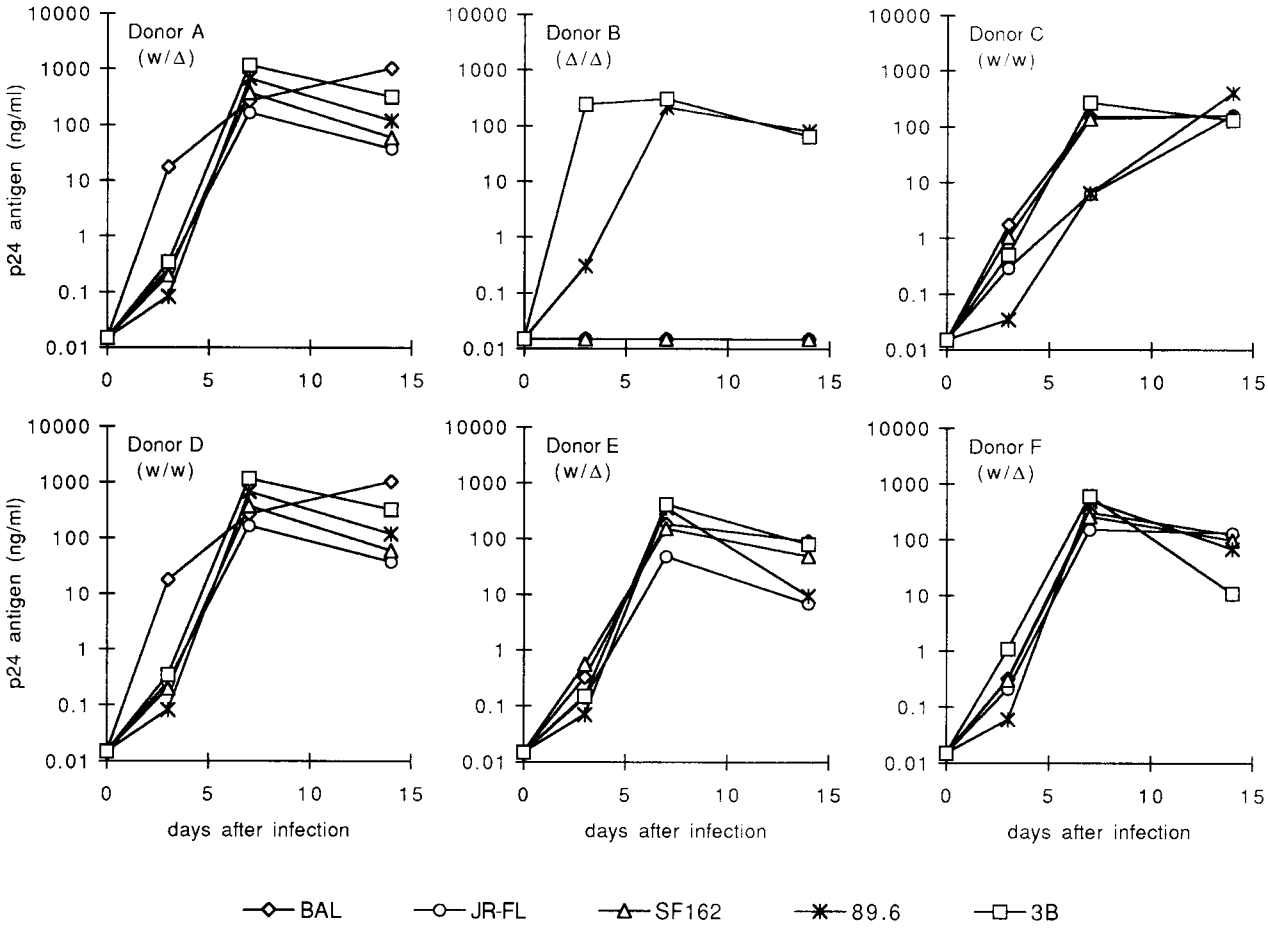

FIG. 2. Replication of prototype HIV-1 strains in primary lymphocytes. PBL were isolated from peripheral blood, depleted of macrophages by serial adherence, and stimulated with PHA followed by IL-2. Cells (4×10^5 per virus) were then infected with M-tropic strains BAL, JR-FL, and SF162; dual-tropic strain 89.6; and T-tropic strain 3B with 25 ng of p24 antigen of each virus. After overnight incubation, they were washed and maintained as described. Supernatant antigen was sampled periodically for p24 antigen production. Donor genotypes are indicated as follows: w/w, homozygous for the wild-type allele; Δ/Δ, homozygous for the Δ*ccr5* mutant allele; and w/Δ, heterozygous. Infections were done in duplicate with similar results.

strains. Three isolates were used that have biological and genetic features typical of M-tropic strains (JR-FL, BAL, and SF162), including tropism for primary macrophages and lymphocytes but not transformed cells, lack of syncytium formation in infected lymphoid cells, and relatively low net charge of the *env* V3 loop region. All three strains have been shown to utilize CCR5 but not CXCR-4 as cofactors for entry and infection in heterologous systems (2, 5, 13–15). We also tested the prototype T-tropic strain 3B, which infects both primary CD4 T cells and transformed $CD4^+$ lines but not macrophages, induces syncytia in infected cells, displays a highly charged V3 loop sequence, and uses CXCR-4 but not CCR5 for entry (14, 16). Finally, we tested a dual-tropic strain, 89.6, that has characteristics of both M-tropic and T-tropic viruses. Like M-tropic strains, it productively infects primary macrophages, but like T-tropic strains it is highly syncytium inducing in lymphocytes, has a relatively high charge V3 loop sequence, and replicates in some transformed cell lines (7). 89.6 can utilize both CXCR-4 and CCR5, as well as the related CCR2b and CCR3 molecules (14).

As shown in Fig. 2, each of the three prototype M-tropic strains failed to replicate in Δ*ccr5* homozygous cells (donor B), while PBL from both heterozygous and normal individuals were readily infected. Thus, not only is CCR5 sufficient to support entry by M-tropic isolates when expressed in nonpermissive cells, but these results indicate that it is also necessary for infection of primary lymphocytes. In these experiments, we could not discern a relationship between the heterozygous genotype and either slower kinetics or peak titers lower than those with wild type. However, these experiments were designed to identify major blocks to infection, and it is likely that differences in relative permissiveness will be best studied with lower inocula and require the analysis of more individuals for statistical comparison. In contrast to the M-tropic strains, the prototype T-tropic isolate 3B readily infected PBL from all donors. The dual-tropic strain 89.6 also infected Δ*ccr5* homozygous cells, consistent with the finding that it can utilize CXCR-4 for entry in transfected cells.

We then determined whether the infection block in homozygous Δ*ccr5* PBL was prior to proviral DNA formation by PCR analysis of cells 3 days after infection (Fig. 3). Reverse transcription products were readily detected in normal PBL after infection with all virus strains, although modest differences in the signal intensities were seen between isolates. In contrast, no PCR signal was seen in Δ*ccr5* homozygous cells after infection with M-tropic strains BAL, JR-FL, and SF162, while 89.6, 3B, and the HXB clone of 3B each resulted in proviral DNA formation. Thus, the M-tropic strains were blocked in PBL that

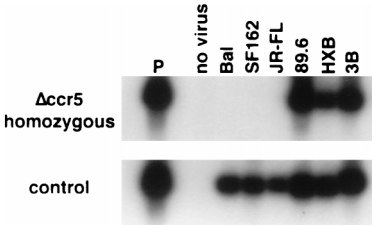

FIG. 3. PCR analysis of proviral DNA formation by prototype HIV-1 strains in primary lymphocytes. PBL from homozygous Δ*ccr5* and normal individuals were stimulated with PHA and IL-2 and infected with DNase-treated virus stock with 25 ng of p24 antigen of each virus. Three days later, they were lysed and subjected to PCR amplification to detect proviral DNA. Plasmid DNA served as a positive control (P).

lack functional CCR5 at an early step of infection, consistent with failure to enter these cells.

Resistance of Δ*ccr5* PBL is not due to CD8 cells or to differences in CD4 expression or viability. CD8 cells can play an important role in limiting HIV replication in mixed PBL cultures and may even underlie the resistance of some individuals to infection in vivo (26, 29). To determine whether the resistance of cells from the Δ*ccr5* homozygous donor B might be due to CD8-mediated virus suppression, we tested whether removal of CD8 cells from PBL cultures would render them susceptible to infection by M-tropic variants. However, the marked restriction of M-tropic variants in Δ*ccr5* homozygous cells was not relieved by CD8 depletion indicating that this is an intrinsic property of the CD4 lymphocytes, although in most donors virus replication was slightly more rapid in CD8-depleted lymphocytes than in mixed PBL cultures (data not shown). We also examined the possibility that differences in cellular CD4 expression might be involved, although this seemed an unlikely cause for such a marked strain-specific block. No differences in CD4 expression among homozygous, heterozygous, and normal donors as measured by flow cytometry were seen (data not shown). Similarly, neither cell viability by trypan blue dye exclusion nor [^{3}H]thymidine incorporation as a measure of cell proliferation differed appreciably either (data not shown).

PBL lacking functional CCR5 do not support infection by primary M-tropic isolates including one that can use CCR3. Because strains present in vivo may differ from prototype viruses, we determined whether the resistance observed with prototype M-tropic viruses would extend to fresh isolates obtained from patients. Conversely, it is also important to determine whether T-tropic strains that emerge in patients can replicate in the absence of functional CCR5 as do laboratory-passaged or prototype T-tropic/SI strains. Therefore, we tested four low-passage primary HIV isolates obtained in our laboratory, selected from a panel of primary isolates because of reciprocal phenotypes. BL-2 and BR-2 are M-tropic/NSI primary isolates that infect primary lymphocytes and macrophages in vitro but fail to replicate in MT-2 or CEMX174 cell lines, while BL-3 and SPL-3 replicate in MT-2 and CEMX174 cells in addition to lymphocytes but poorly or not at all in macrophages (data not shown). In these experiments, cells from an additional donor homozygous for the Δ*ccr5* mutation were studied. As shown in Fig. 4, the T-tropic strains BL-3 and SPL-3 infected lymphocytes from all four donors, while the M-tropic primary isolates BL-2 and BR-2 replicated in cells from normal but not Δ*ccr5* homozygous donors. This indicates that the pattern identified with prototype M- and T-tropic viruses extends to variants derived from patients as well.

To further examine HIV variants present in vivo, we also tested strain YU-2, which is biologically similar to other M-tropic/NSI strains and was cloned directly from tissue (and was therefore not subject to selective pressures in vitro during isolation) (23). Interestingly, YU-2 is one of a subset of M-tropic isolates that can use the related molecule CCR3 in addition to CCR5 (5). In Δ*ccr5*-homozygous PBL, however, YU-2 also failed to replicate (Fig. 4), indicating that it too required CCR5 and that CCR3 did not substitute as a cofactor for entry into primary lymphocytes.

Of note, in some experiments low titers of viral antigen (i.e., ~0.2 ng/ml) were seen after infection of Δ*ccr5* homozygous cells by some M-tropic isolates, including both primary and prototype strains (Fig. 4 and data not shown). Although this finding was inconsistent and may reflect residual inoculum, a very limited, highly restricted infection cannot be completely ruled out (29).

Primary macrophages lacking CCR5 fail to support prototype M-tropic HIV-1. The importance of M-tropic variants in person-to-person spread suggests that infection of macrophages or related cells may be a critical step in virus transmission. In addition, macrophages and related cells play an important role in the development of neurologic and pulmonary complications of infection. To determine whether CCR5 is the main HIV-1 entry cofactor on macrophages as it is for M-tropic strains on lymphocytes, we tested whether primary macrophages lacking functional CCR5 were also resistant to infection. Monocytes were isolated from PBMC, allowed to differentiate into macrophages, and infected with the prototype M-tropic strain SF162. As shown in Fig. 5, sustained levels of p24 antigen were produced by macrophages from five of six donors. In four of these (two normal and two heterozygous) the infection was highly productive (peak levels of ≥50-ng/ml p24), while cells from one heterozygous individual produced only modest antigen levels. In contrast, macrophages from the homozygous Δ*ccr5* donor B were essentially resistant to SF162. Large multinucleated giant cells that were characteristic of productively infected macrophages (8) were also prominent in cultures from donors A, C, D, and E but absent in those from B and F (data not shown). Thus, CCR5 is the principal coreceptor for infection of macrophages as well as lymphocytes.

Infection of Δ*ccr5* macrophages by strains that utilize multiple cofactors. To determine whether CCR5 was needed for macrophage infection by a broader range of isolates, we then tested several additional strains including JR-FL, YU-2, and 89.6 (Fig. 6). We focused on these viruses because CCR5 is the only cofactor so far identified for JR-FL, while YU-2 can use both CCR5 and CCR3, and 89.6 can use CCR5, CCR3, and CCR2b as well as CXCR-4 (5, 14). As expected, JR-FL failed to replicate in macrophages lacking CCR5. Similarly, YU-2 was also unable to replicate in these cells, indicating that CCR3 does not serve as a cofactor for entry into primary macrophages. Interestingly, however, 89.6 was able to infect macrophages in the absence of functional CCR5. Because T-tropic, CXCR-4-dependent strains are by definition unable to enter and infect macrophages it is unlikely that CXCR-4 is the mechanism used by 89.6 for entry, which suggests that either CCR2b or some other related molecule is serving this function.

DISCUSSION

The viral determinants of HIV-1 target cell tropism mainly map to *env*, in agreement with the observation that tropism is determined largely by viral entry (19, 20, 27, 36, 41). Recently, the chemokine receptors CCR5 and CXCR-4 have been identified as cofactors that, in conjunction with CD4, allow fusion and entry of M-tropic and T-tropic strains, respectively, into

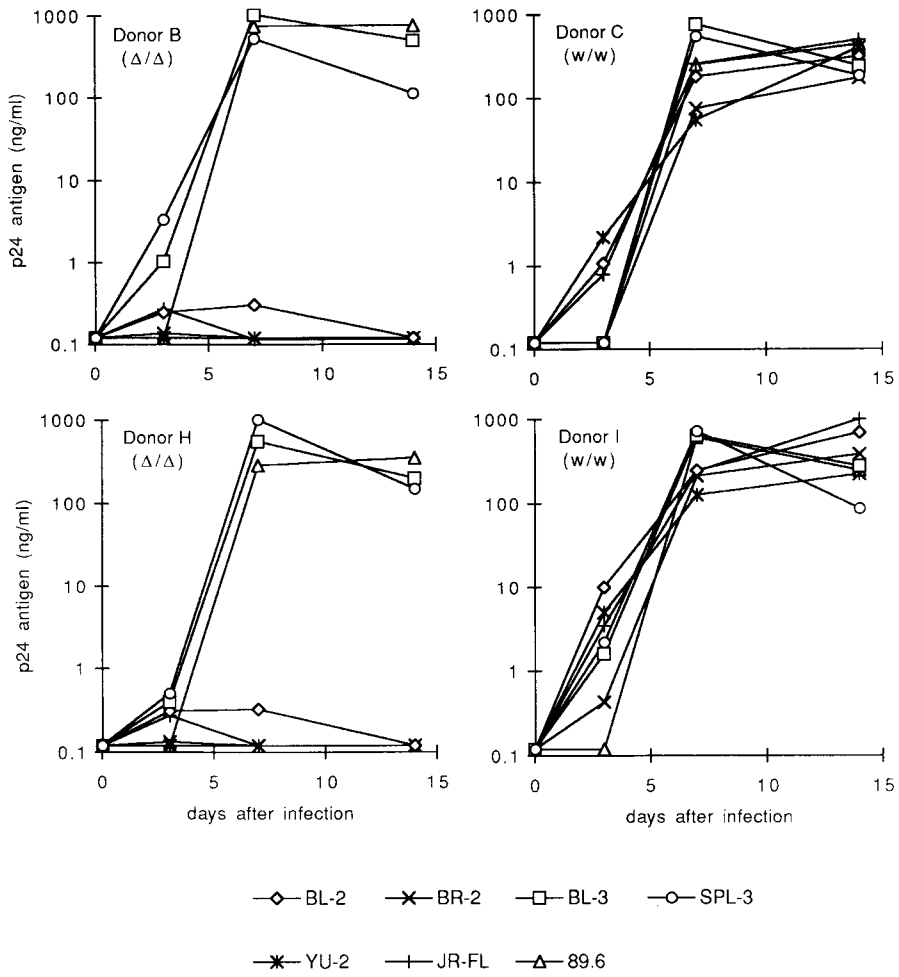

FIG. 4. Replication of primary HIV-1 isolates in lymphocytes. PBL were isolated and maintained as described in the legend to Fig. 2. Cells were then infected as described with two T-tropic/SI primary isolates (BR-3 and SPL-3) and three M-tropic/NSI primary isolates (BL-2, BR-2, and YU-2) by using 25 ng of p24 antigen of each virus and were sampled periodically. Infections were done in duplicate with similar results, and donor genotypes are as indicated in Fig. 2.

otherwise nonpermissive heterologous cells (2, 5, 13–16). CCR5 binds MIP-1α, MIP-1β, and RANTES (31), the three chemokines identified as responsible for CD8 cell inhibition of infection by M-tropic but not T-tropic variants (6, 29). Similarly, stromal cell-derived factor 1 (SDF-1) is the recently identified ligand for CXCR-4, and it inhibits infection by T-tropic strains (3, 28). Some isolates can use other CC chemokine receptors such as CCR3 and CCR2b as well (5, 14). The selective use of cofactor molecules for HIV-1 entry provides a basis for the cellular determinants of target tropism. Sorting out how CCR5, CXCR-4, and other cofactors are used by HIV in vivo is important for understanding many aspects of pathogenesis, including viral transmission, phenotypic evolution, brain and lung infections, and the natural regulation of virus by chemokines, and will also form the basis for the development of therapeutic strategies designed to target viral entry.

The Δ*ccr5* mutation confers protection against HIV-1 infection in vivo (12, 24, 32). Using an antiserum to native CCR5, we found that Δ*ccr5* expression results in protein that can be detected only intracellularly, in contrast to wild-type CCR5, which is expressed on the cell surface. While we utilized an avian expression system and it is possible that the processing or targeting of specific proteins may differ between avian and mammalian cells, our results are consistent with those reported previously with an epitope-tagged Δ*ccr5* that was detected with a monoclonal antibody directed against the tag, in which protein could not be detected at the surface of transfected mammalian cells (24). The 32-bp deletion in Δ*ccr5* predicts a molecule that is prematurely terminated within the third extracellular domain with the loss of regions critical for G-protein-coupled signal transduction (32, 38). While the N-terminal and second extracellular domains of CCR5 largely determine the specificity of cofactor use (30) and are retained in the truncated molecule, all of the regions required for HIV cofactor function are not yet known. However, the failure of Δ*ccr5* protein to reach the cell surface obviously results in a protein without HIV cofactor activity.

CCR5 can serve as a coreceptor for entry of M-tropic isolates when introduced along with CD4 into otherwise nonpermissive cells, but the broad nature of cofactor usage by some HIV-1 strains suggests that there may also be other not yet identified related molecules that serve to support virus entry (5, 14). In addition, while the chemokine ligands to CCR5 block fusion and infection by M-tropic variants, considerable overlap exists among β-chemokines in their receptor-binding specificity (33), and primary lymphocytes and macrophages are substantially less sensitive to the protective effects of the MIP-1α, MIP-1β, and RANTES than are transfected cells or PM-1

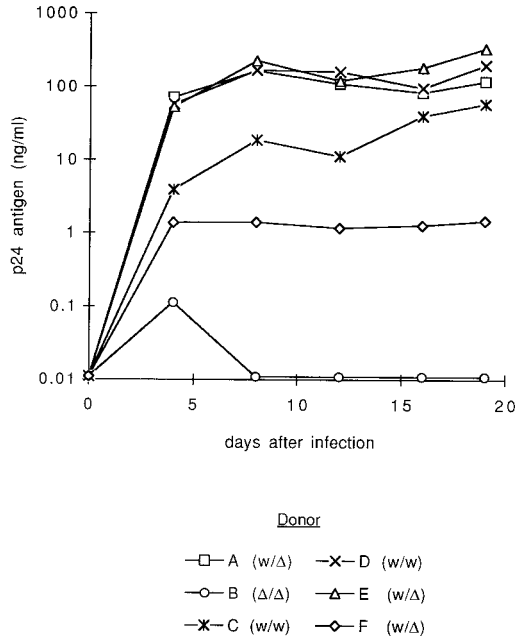

FIG. 5. Infection of primary monocyte-derived macrophages. Macrophages were isolated from PBMC by plastic adherence as described in Materials and Methods and allowed to differentiate in vitro. They were then infected overnight with M-tropic strain SF162 (60 ng of p24 antigen), washed, and refed, and supernatant was sampled periodically. Donor genotypes are as indicated in the legend to Fig. 2.

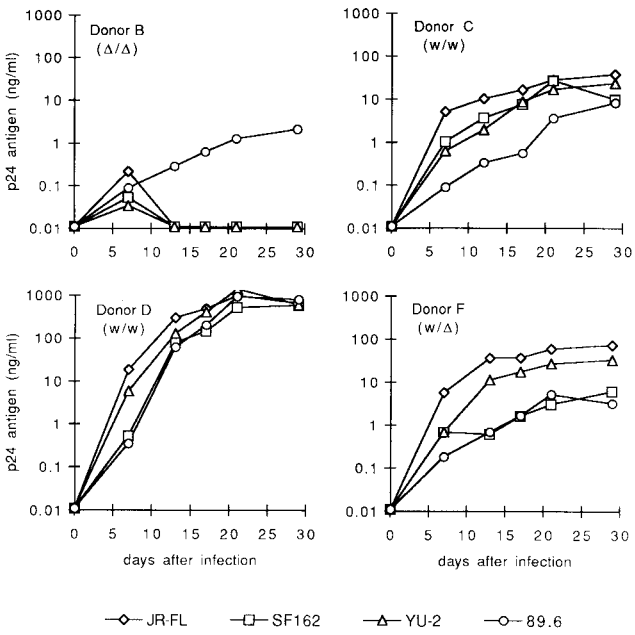

FIG. 6. Replication of different M-tropic isolates in primary macrophages. Macrophages were isolated from PBMC by selective adherence as described in Materials and Methods and were allowed to differentiate in vitro. They were then infected with M-tropic strains JR-FL, SF162, and YU-2 or with dual-tropic isolate 89.6 by using 25 ng of p24 antigen of each virus. After overnight incubation, they were washed and refed, and supernatant was sampled periodically for p24 antigen production. Data shown are representative of duplicate infections which gave similar results. Donor genotypes are as indicated in the legend to Fig. 2.

cells (6, 15, 34), raising the possibility that CCR5 may be one of several molecules that support HIV entry in its natural target cells. Δ*ccr5* cells provide a naturally occurring knockout model to address these questions. The finding that CCR5 is required for efficient HIV-1 infection of primary macrophages and lymphocytes by most prototype as well as patient-derived M-tropic strains is important, because it shows that CCR5 is not only sufficient to serve as an entry cofactor but is necessary and that it is the principal coreceptor for entry in the two major target cells infected by HIV-1 in vivo. However, it cannot be ruled out that the extremely low levels of antigen seen after infection of CCR5-negative cells in some experiments might reflect very low-efficiency infection by some other pathway.

Several strains, including YU-2 and 89.6, can also enter target cells via CCR3 (5, 14). We found that YU-2 was unable to replicate in Δ*ccr5* homozygous lymphocytes or macrophages, indicating that CCR3 does not function as a cofactor for entry in these target cells. This result is not unexpected, since CCR3 is expressed mainly in eosinophils and not in monocytes or lymphocytes (9). While their potential role in pathogenesis is unknown, HIV-1 has been reported to infect eosinophils (18, 40), and it is conceivable that CCR3 or other cofactors with limited distributions may play a role in that regard. Of further interest is CCR2b, which is highly expressed on monocytes (9, 10) and supports entry by the dual-tropic isolate 89.6. This strain also uses CCR5, CCR3, and CXCR-4 (14). Not unexpectedly, 89.6 replicated efficiently in lymphocytes lacking CCR5, probably on the basis of CXCR-4-mediated entry. However, 89.6 also replicated in macrophages that lack CCR5. CXCR-4-dependent T-tropic viruses by definition fail to infect primary macrophages, and envelope proteins derived from these isolates do not fuse with primary macrophages (1). It is thus unlikely that CXCR-4 is responsible for 89.6 entry into Δ*ccr5* macrophages, although we cannot rule out the possibility that cofactors may be utilized differently by different strains. This suggests that a cofactor other than CCR3 or CXCR-4 is responsible for 89.6 infection in the absence of functional CCR5. CCR2b is a possible candidate, given its distribution. However, in preliminary studies we have not been able to block 89.6 infection of Δ*ccr5* macrophages with monocyte chemoattractant protein 1 (MCP-1) and MCP-3 (data not shown), which are the chemokine ligands for CCR2b. While it is conceivable that the ability of chemokines to block infection mediated by their respective receptors may differ for the various cofactors, the promiscuous nature of 89.6 cofactor usage also raises the possibility that there are other cofactor molecules that support entry into primary macrophages as well. Additional studies are required to determine whether CCR2b and/or another molecule(s) is responsible for this infection, and to further define the range of isolates that can enter macrophages in the absence of CCR5.

The two individuals previously reported whose lymphocytes were resistant in vitro to M-tropic isolates and who were found to be homozygous for the Δ*ccr5* allele were identified because they remained uninfected despite repeated exposure to HIV (24). More recently, macrophages derived from these individuals were also found to resist infection by several M-tropic isolates (11). In our study, we tested cells from donors who were identified by random screening of laboratory workers rather than from a highly selected group known to be resistant to infection. The fact that these cells, unselected for in vivo exposure or resistance, could not be productively infected with several different M-tropic strains provides compelling evidence that lack of functional CCR5 itself is responsible for resistance to M-tropic strains at the cellular level. The convergence of data showing that M-tropic isolates are involved in person-to-

person transmission, that CCR5 can serve as a coreceptor for infection by M-tropic strains, and that individuals lacking CCR5 are protected against infection, is strengthened by our findings that CCR5 is not only sufficient but also necessary for infection of two major relevant primary target cells by patient-derived as well as prototype M-tropic isolates. While M-tropic isolates have a critical role in person-to-person spread, it remains unknown whether this is due to the selective presence of M-tropic variants in donor secretions, the need to infect macrophages or macrophage-related cells in recipient mucosa for successful transmission, or some other characteristic of M-tropic variants such as the NSI phenotype or relative resistance to neutralization. It will also be very important to define the role of CCR5 and other cofactors in infection of other primary cell types such as dendritic cells that are important targets for infection and pathogenesis in vivo (17, 37).

The reciprocal patterns of CCR5 and CXCR-4 usage by M-tropic and T-tropic strains, respectively, suggest a simple model for the cellular determinants of tropism in which CCR5 is expressed by macrophages, CXCR-4 is expressed by transformed cells lines, and both are expressed by lymphocytes. However, CXCR-4 was initially cloned from a cDNA library derived from primary human macrophages where it appears to be expressed (25), and SDF-1, the recently identified ligand for CXCR-4, induces calcium currents on human monocytes (28). Thus, if CXCR-4 is indeed expressed by macrophages, it will be important to determine whether levels of expression, differential processing, or the presence of other molecules may affect its ability to function as a cofactor for HIV in macrophages.

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