

Coreceptor Utilization by Human Immunodeficiency Virus Type 1 Is Not a Primary Determinant of Neutralization Sensitivity

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Received 24 September 1997/Accepted 4 December 1997

We have examined the relationship between coreceptor utilization and sensitivity to neutralization in a primary isolate of human immunodeficiency virus type 1 and its T-cell line-adapted (TCLA) derivative. We determined that adaptation of the primary-isolate (PI) virus 168P results in the loss of the unique capacity of PI viruses to utilize the CCR5 coreceptor and in the acquisition by the TCLA 168C virus of sensitivity to neutralization by V3-directed monoclonal antibodies (MAbs). In experiments wherein infection by 168P is directed via either the CCR5 or the CXCR4 pathway, we demonstrate that the virus, as well as pseudotyped virions bearing a molecularly cloned 168P envelope protein, remains refractory to neutralization by MAbs 257-D, 268-D, and 50.1 regardless of the coreceptor utilized. This study suggests that coreceptor utilization is not a primary determinant of differential neutralization sensitivity in PI and TCLA viruses.

Although CD4 had long been recognized as the cellular receptor to which the human immunodeficiency virus type 1 (HIV) envelope protein binds (9, 21, 22), it had also been recognized that expression of CD4 alone is insufficient to render nonhuman cells susceptible to HIV infection (4, 5, 22). Similarly, different HIV isolates display different abilities to infect CD4-positive human macrophages, T lymphocytes, and established T-cell lines (31, 32, 35), suggesting that additional molecules may be responsible for cell tropism specificity. During the past year, cellular molecules that act in conjunction with CD4 have been identified as required cofactors for HIV envelope protein-mediated binding and entry (1, 6, 10–12, 14). These HIV coreceptors are members of the superfamily of seven-transmembrane segment G-protein-coupled receptors and act primarily as cellular receptors for chemokines.

The discovery of cellular coreceptors for HIV has provided new perspectives for understanding these early events in HIV infection (see review in reference 2). Thus, phenotypically distinct isolates of HIV utilize as coreceptors different chemokine receptor molecules. Although all primary isolates of HIV infect primary T lymphocytes, some also infect cells of the macrophage lineage (31, 32). These monocytotropic isolates utilize the CCR5 chemokine receptor, whose natural ligands include the chemokines RANTES, MIP-1 α , and MIP-1 β (1, 6, 10–12). Monocytropic isolates do not induce syncytia in primary lymphocyte culture and do not infect established T-cell lines (31). During the late course of HIV infection, syncytium-inducing (SI) primary viruses often arise from the population of monocytotropic viruses (31, 32). These SI primary isolates no longer infect macrophages, and they utilize both CCR5 and another chemokine receptor, CXCR4 (7, 33, 38). CXCR4, whose natural chemokine ligand is SDF-1 (3, 27), was originally identified by Feng et al. as the cofactor used by laboratory-adapted

viruses (14). In fact, the common laboratory viruses (IIIb/LAI, LAV, and RF) are unable to utilize CCR5 coreceptor (1, 6, 10–12), presumably reflecting the lack of CCR5 expression in most established T-cell lines (1, 13). Although some primary isolates utilize additional chemokine receptor molecules, notably CCR3 and CCR2b (6, 11, 18), the relationship between these coreceptors and viral phenotypes is less clear. The ability to utilize CCR5 coreceptor, however, is unique to primary-isolate (PI) viruses.

Paralleling these differences in coreceptor utilization and cell tropism are differences in sensitivity to virus neutralization. Although laboratory-adapted isolates of HIV can be potently neutralized by sera elicited by recombinant gp120 (rgp120) protein, primary isolates are largely refractory to neutralization by rgp120 vaccine sera (23, 37). Similarly, PI viruses are significantly more resistant than T-cell line-adapted (TCLA) viruses to neutralization by gp120-directed monoclonal antibodies (MAbs) (25, 37) and to inhibition by soluble forms of CD4 (8). We and others have demonstrated that neutralization sensitivity develops concomitantly with adaptation of primary isolates to persistent growth in established T-cell lines (24, 37). By studying pedigreed PI and TCLA viruses (168P and 168C, respectively), we have shown that adaptation renders the TCLA virus sensitive not only to rgp120 vaccine sera and CD4 immunoadhesin but also to MAbs directed to the V3 loop of gp120 (37). However, the basis for this increase in neutralization sensitivity remains unclear.

In this report, we explore the relationship between neutralization sensitivity and coreceptor utilization, especially with regard to changes that accompany adaptation. We examined neutralization sensitivity of the well-characterized SI primary isolate 168P under experimental conditions where infection can be directed via either the CXCR4 or the CCR5 pathway. The pedigreed TCLA derivative 168C utilizes only CXCR4 and was sensitive to neutralization by the panel of V3-directed MAbs used in these assays. However, the primary isolate 168P remained refractory to neutralization regardless of coreceptor pathway taken. Our findings suggest that envelope protein

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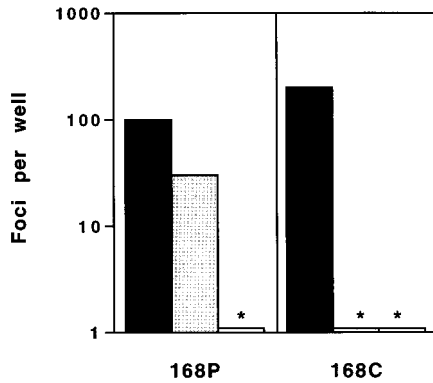


FIG. 1. Coreceptor utilization by pedigreed PI and TCLA 168 viruses. U87-CD4 cell lines expressing CXCR4 (■) or CCR5 (▨) were used to define the ability of 168P and 168C viruses to utilize the respective coreceptor. CCR5 utilization was further tested by the addition to U87-CD4-CCR5 cells of CCR5-specific chemokines (RANTES, MIP-1 α , and MIP-1 β ; R&D Systems) (□). For details, see text. *, no foci were observed.

structure, and not coreceptor utilization, is the primary determinant of differential neutralization sensitivity in PI and TCLA viruses.

Coreceptor utilization by pedigreed PI and TCLA viruses.

Cross-sectional surveys of coreceptor use have shown that primary SI isolates generally utilize CXCR4 and CCR5 coreceptors, whereas unrelated laboratory-adapted isolates utilize only CXCR4 (1, 6, 7, 10–12, 14, 33, 38). We wished to confirm this trend in a longitudinal study of adaptation. We previously described the adaptation of the SI primary isolate 168P to persistent growth in the FDA/H9 T-cell line and the concomitant development of neutralization sensitivity in the resulting TCLA virus 168C (37). In the present study, the ability of these pedigreed viruses to utilize specific coreceptors was tested by infection of U87 human glioma cell lines expressing CD4 (U87-CD4) and the specific coreceptor (19).

For this assay, virus stocks were prepared from cell culture supernatants of phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBLs) (168P) or FDA/H9 cells (168C) and standardized to yield a submaximal number of foci of infection on U87-CD4-CXCR4 cells (approximately 100 to 200

foci/96-well microplate culture). To confirm coreceptor specificity, in some assays CCR5 chemokines (each at 500 ng/ml) were added to cells 1 h prior to infection. After 2 days of incubation, cell monolayers were fixed with methanol-acetone and immunochemically stained with HIV immunoglobulin (HIVIG) (29), anti-human ABC kit (Biomedica Corp.), and diaminobenzidine substrate.

Figure 1 confirms the ability of the SI 168P virus to utilize both CXCR4 and CCR5 and the subsequent loss of this latter specificity in the 168C TCLA virus. Infection was dependent on coreceptor expression, and both PI and TCLA viruses could also utilize CCR3 (data not presented).

In keeping with the determined coreceptor specificity, infection could be blocked by addition of coreceptor-specific ligands. Thus, 168P virus infection of CCR5-expressing cells was blocked by the CCR5-specific ligands RANTES, MIP-1 α , and MIP-1 β (1, 6, 10–12) (Fig. 1). Similarly, infection of CXCR4-expressing U87-CD4 cells by either virus could be blocked by the CXCR4-specific chemokine ligand SDF-1 (3, 27) (data not presented).

Coreceptor pathway and neutralization sensitivity. In previous work, we demonstrated that the PI 168P virus is refractory to neutralization by HIV MN gp120 vaccine sera and by several well-characterized V3-directed murine MAbs which strongly neutralize infectivity of the TCLA 168C virus (37). In the present study, we extended the panel of MAbs to include two V3-directed human MAbs, 257-D and 268-D (17). These well-characterized human MAbs recognize core epitopes at the crown of the V3 loop of gp120 (KRIHI and HIGPGR, respectively), linear sequences known to be present in both 168P and 168C envelope proteins (37). These epitope predictions were confirmed by gp120 capture enzyme-linked immunosorbent assay (ELISA) (26) which demonstrated equal binding to envelope protein in detergent-solubilized 168P and 168C virions (data not presented). Sensitivity to neutralization by these human MAbs was determined in a standard assay using PHA-activated PBLs (37). MAbs 257-D and 268-D were found to potently neutralize 168C but fail to neutralize 168P (Fig. 2). This pattern of neutralization sensitivity is similar to that previously described for the V3-directed murine MAb 50.1 (30, 36, 37).

To examine whether sensitivity to neutralization was af-

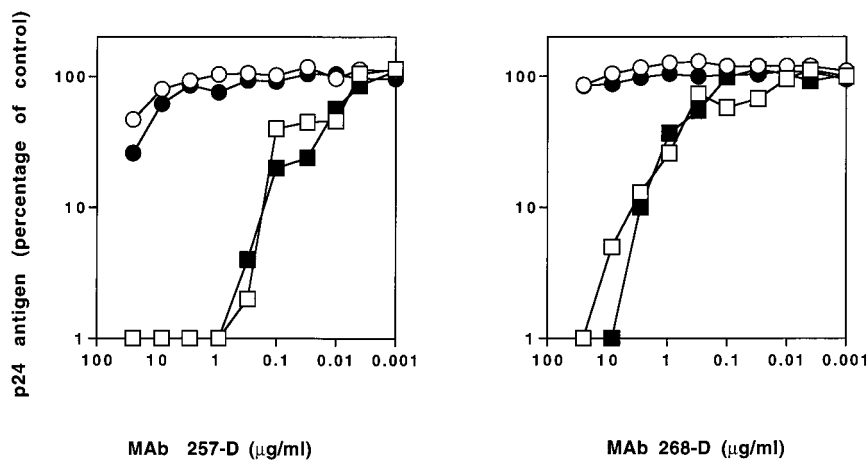


FIG. 2. Neutralization sensitivity of 168 viruses in PBL culture. Virus neutralization assays in PHA-stimulated PBL culture were performed as previously described (37). 168P (○, ●) and 168C (□, ■) virus stocks were standardized to yield submaximal extents of virus spread during the 5-day infection. CCR5-specific chemokines (●, ■) were added as described for Fig. 1. The V3-directed MAbs are indicated. p24 antigen was determined by p24 antigen capture ELISA (SAIC Frederick) and was normalized to infected cell control values (168P, 190 ng/ml [170 ng/ml with chemokines]; 168C, 36 ng/ml [33 ng/ml with chemokines]).

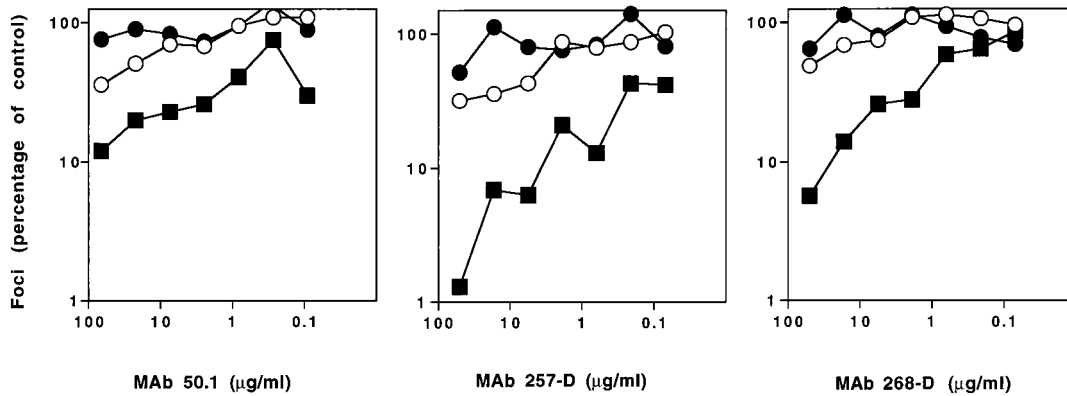


FIG. 3. Neutralization sensitivity of 168 viruses in U87-CD4 cell lines expressing CCR5 or CXCR4 coreceptor. 168P (○, ●) and 168C (■) viruses were used to infect U87-CD4 cell lines expressing CXCR4 (●, ■) or CCR5 (○) as described for Fig. 1. The V3-directed MABs were incubated with virus for 1 h prior to infection.

ected by the coreceptor pathway utilized in infection of PBLs, we used inhibitory concentrations of CCR5-specific chemokine ligands RANTES, MIP-1 α , and MIP-1 β in order to restrict infection to the CXCR4 pathway. Addition of these chemokines to the PBL cultures did not affect virus growth, nor did it affect sensitivity to neutralization by the V3-directed human MABs (Fig. 2). To the extent that CCR5 blockade was complete, these results suggest that the simple availability of the CCR5 pathway is not a factor in the resistance of PI viruses to neutralization.

To strengthen this conclusion, we examined neutralization sensitivity in human U87-CD4 cell lines expressing only CXCR4 or CCR5. Using this method, we confirmed that the SI 168P virus remained refractory to neutralization by human MABs 257-D and 268-D as well as by the murine MAB 50.1, regardless of whether infection occurred via CXCR4 or CCR5 (Fig. 3). These results suggest that availability of the CCR5 pathway is not a primary determinant for the resistance of PI viruses to neutralization. The TCLA 168C virus utilized CXCR4 only and was sensitive to neutralization.

Molecularly cloned PI and TCLA envelope genes. To understand better the changes that accompany adaptation and those that determine coreceptor utilization and neutralization sensitivity, we molecularly cloned the envelope genes of the 168P and 168C viruses. High-fidelity XL PCR (rTth and Vent DNA polymerases; PE Applied Biosystems) and primers envA and envN (15) were used to amplify a 3.1-kb region of proviral DNA encoding the *rev* and envelope genes. PCR products were isolated by unidirectional T/A cloning in the eucaryotic expression vector pCR3.1-Uni (Invitrogen). Expression in pCR3.1-Uni is driven by the cytomegalovirus immediate-early promoter. Multiple clones were isolated from each virus, and transient transfection studies in COS-7 cells confirmed the surface expression and fusion competence of all clones tested (data not presented).

DNA sequence analysis demonstrated that all 168C molecular clones analyzed encoded the three adaptation-associated amino acid changes previously identified by PCR sequencing of the 168C virus population (V2, I166R; C2, I282N; and V3, G318R) (37). Two molecular clones of each 168P and 168C envelope were subjected to complete DNA sequence analysis (GenBank accession no. AF035532 to AF035534). Molecular clones 168C23 and 168C60 were identical throughout the envelope gene. Molecular clones 168P5 and 168P23 differed from each other and from the previously determined sequence at four to five positions distinct from those associated with adaptation. These scattered changes within the primary virus quasi-

species are considered inconsequential at the present level of analysis; the significance of the three adaptation-associated changes is under separate investigation.

Functional analysis of these molecularly cloned envelope genes was performed by incorporation of the molecularly cloned envelope protein into pseudotyped HIV virions. We used an envelope-defective provirus derived from the molecularly cloned NL4-3 provirus (kindly provided by I. S. Y. Chen, University of California, Los Angeles). The pNLthy Δ Bgl provirus (28) contains a *Bgl*II-*Bgl*III deletion within the envelope gene and a substitution of the viral *nef* gene with a cDNA encoding the murine Thy1.2 cell surface protein. The simian virus 40 *ori* was subsequently introduced into the plasmid to generate pSVNLthy Δ Bgl (27a). Cotransfection of COS-7 cells (16, 20) with pSVNLthy Δ Bgl provirus and the envelope expression plasmid resulted in the production of pseudotyped HIV virions. Culture supernatants were harvested 3 days posttransfection, filtered, and used to infect U87-CD4 cell lines expressing coreceptor. Cells infected by virions bearing the complementing envelope protein were identified by immunostaining for murine Thy1.2 or HIV proteins.

As anticipated, the molecularly cloned envelope proteins recapitulated the coreceptor specificity of the parental virus population (see the legend to Fig. 4). Pseudotyped virions containing 168C60 were able to infect only U87-CD4 cells expressing CXCR4, while virions containing 168P23 envelope were able to infect U87-CD4 cells expressing either CCR5 or CXCR4. Thus, the viral envelope protein appears to be the major, if not sole, determinant of viral coreceptor use. These findings also indicate that dual coreceptor use is a direct property of the envelope protein complex and not a result of a mixture of distinct envelope proteins in the SI virus population. This conclusion is corroborated by the failure of CCR5-specific chemokine ligands to diminish 168P virus infection in PBL culture (Fig. 2).

Finally, we wished to determine the neutralization sensitivity of pseudotyped virions containing the molecularly cloned 168P23 and 168C60 envelope proteins and to confirm that coreceptor pathway is not a primary determinant of neutralization sensitivity. We found that infection of U87-CD4-CXCR4 cells by pseudotyped virions containing 168C60 envelope protein was sensitive to neutralization by MABs 257-D, 268-D, and 50.1 at concentrations comparable to those determined in assays using 168C virus (Fig. 4). Pseudotyped virions containing 168P23 envelope protein remained refractory to neutralization by all three V3-directed MABs, regardless of the coreceptor expressed by the U87-CD4 cell line.

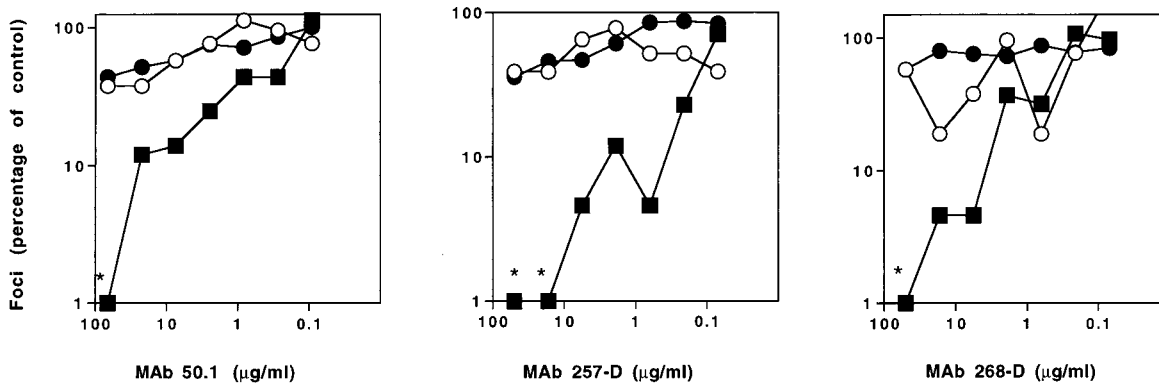


FIG. 4. Neutralization sensitivity of pseudotyped virions in U87-CD4 cell lines expressing CCR5 or CXCR4 coreceptor. Pseudotyped virions were derived by cotransfection of COS-7 cells with pSVNLthy Δ Bgl provirus and plasmid expressing 168P23 (○, ●) or 168C60 (■) envelope protein. Virion preparations were incubated with U87-CD4 cell lines expressing CXCR4 (●, ■) or CCR5 (○) as described for Fig. 1; V3-directed MAbs were added as indicated. The number of foci was normalized to control values (60 to 100 foci/well for U87-CD4-CXCR4 cells; 10 foci/well for U87-CD4-CCR5 cells). *, no foci were observed.

In summary, we examined the relationship between coreceptor utilization and sensitivity to neutralization by V3-directed MAbs. The observed dichotomy in the sensitivity to neutralization of PI and TCLA viruses had suggested a discrete difference between these viruses, and we tested one hypothesis: that PI viruses are refractory to neutralization as a result of their unique ability to utilize the CCR5 coreceptor. We examined neutralization sensitivity of a well-characterized SI primary isolate under experimental conditions wherein the virus was forced to utilize either CCR5 or CXCR4 for infection. We showed that coreceptor pathway is not a direct determinant of neutralization sensitivity. The primary virus envelope protein remained refractory to neutralization by V3-directed MAbs regardless of the coreceptor pathway utilized. Similarly, coreceptor utilization did not affect neutralization sensitivity by soluble CD4 (34) or HIVIG (data not presented).

In discarding the otherwise attractive hypothesis that PI viruses escape neutralization through their unique ability to utilize CCR5, we are left to consider the as yet undefined structural differences between the envelope protein complex of PI and TCLA viruses. Several studies have suggested that critical determinants in the envelope protein of PI viruses are less accessible than those of TCLA viruses and that it is this differential access that determines neutralization sensitivity (reviewed in reference 25). By contrast, our studies have indicated similar binding of V3-directed MAbs to PBLs infected with neutralization-resistant isolate 168P or neutralization-sensitive isolate 168C (37). Thus, the basis for the differential neutralization sensitivity of PI and TCLA viruses remains unresolved.

Our present studies also do not address whether changes in coreceptor utilization and/or neutralization sensitivity are necessarily linked as a consequence of adaptation. The analysis of independently derived PI and TCLA viruses may allow further separation of these viral phenotypes. Subsequent dissection of the amino acid changes that distinguish pedigreed PI and TCLA envelope proteins will help to define the structural bases underlying the changes that accompany adaptation.

This work was supported by NIH AREA grant AI41165 to J.H.N. and by NIH grants AI32424 and AI36085 to S.Z.-P. Additional funds were provided by The University of Montana, the M. J. Murdock Charitable Trust, and the Department of Veterans Affairs.

We thank Ed Berger (NIH) and Ned Landau (Aaron Diamond AIDS Research Center) for useful discussions during the course of these studies. We are also grateful to numerous colleagues who con-

tributed reagents for this work: Dan Littman (HHMI, NYU Medical Center), Thera Mulvanica and Jim Mullins (University of Washington) for U87-CD4 cell lines, Irvin Chen (UCLA School of Medicine) for pNLthy Δ Bgl provirus, Terri Wrin (Genentech, Inc.) for 168P and 168C viruses, Ian Clark-Lewis (University of British Columbia) for SDF-1, Steve Chamow (Genentech, Inc.) for recombinant CD4-Ig, and Fred Prince (New York Blood Center) for HIVIG. Oligonucleotides and DNA sequence analysis were provided by Joan Strange at The University of Montana Murdock Molecular Biology Laboratory. We thank John Moore (Aaron Diamond AIDS Research Center) for assistance in the ELISA determination of MAb binding. The NIH AIDS Research and Reference Reagent Program provided initial samples of CCR5-specific chemokines, HIVIG, and MAbs 50.1, 257-D, and 268-D, as well as several reagents which were not expressly represented in these studies but which were nonetheless important.

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