Role of Low CD4 Levels in the Influence of Human Immunodeficiency Virus Type 1 Envelope V1 and V2 Regions on Entry and Spread in Macrophages

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Human immunodeficiency virus type 1 (HIV-1) isolates vary in their ability to infect macrophages. Previous experiments have mapped viral determinants of macrophage infectivity to the V3 hypervariable region of the HIV-1 envelope glycoprotein. In our earlier studies, V1 and V2 sequences of HIV-1 were also shown to alter the ability of virus to spread in macrophage cultures, whereas no effect was seen in lymphocyte cultures. In the present study, determinants that allowed certain HIV-1 clones to infect and spread in macrophages were primarily mapped to the V2 region and were found to act by influencing early events of viral infection. In an assay of viral entry into macrophages, it was shown that viruses with the V2 region from the Ba-L strain of HIV-1 had >10-fold-higher entry efficiency than viruses with the V2 region derived from the NL4-3 strain. V1 region differences between these groups caused a twofold difference in entry. The known low expression of CD4 on macrophages appeared to be important in this process. In entry assays conducted with HeLa cell lines expressing various levels of CD4 and CCR5, low levels of CD4 influenced the efficiency of entry and fusion which were dependent on viral V1 and V2 envelope sequences. In contrast, no effect of V1 or V2 was seen in HeLa cells expressing high levels of CD4. Thus, the limited expression of CD4 on macrophages or other cell types could serve as a selective factor for V1 and V2 envelope sequences, and this selection could in turn influence many aspects of AIDS pathogenesis in vivo.
the low levels of CD4 that are expressed on macrophages (15, 24, 29, 44), viral entry studies were also conducted with cell lines expressing different levels of the CD4 and CCR5 viral receptors (35). These experiments provided evidence that low CD4 levels were a significant barrier to entry of cells by HIV-1 clones with certain V1/V2 sequences. By acting as a selective factor on viruses during infection of macrophages in brain and other tissues of AIDS patients, this mechanism may influence various aspects of pathogenesis and disease progression.

MATERIALS AND METHODS

Construction of recombinant HIV-1 clones. Construction of clone 81A with the Ba-L strain V1, V2, and V3 regions (designated BBG) in the background of strain NL4-3 (1) and clone 49-5 with the NL4-3 strain V1 and V2 regions, followed by Ba-L sequences from Stul to NheI encompassing the C2 and V3 regions (designated NNB) in the same background NL4-3 strain were described previously (8, 11, 43). To be able to replace V1 and V2 region sequences independently, additional clones were generated for this study as follows: clone 49-5 was digested with Ba-L and BamHI linkers and inserted to remove this unique PsiAI site. An oligonucleotide polylinker with sites EcoRI-Sall-PsiAI of sites EcoRI and Stul to generate clones 46-30. Next, the NL4-3 V1 region plus sequences encoding 18 upstream residues plus 8 residues of V2 from NL4-3 were inserted into these plasmids to replace sequences removed earlier in clone 46-30. Three NL4-3 strain V1 region plus sequences encoding V2 sequences were inserted as described above. Clone 146-7 contains upper-strand and three lower-strand oligonucleotides as described previously containing sequences from either NL4-3 or Ba-L by annealing a complex of three upper-strand oligonucleotide 1393 (5'-TGG GAC CAA AGT CTA AAG CCA TGT G-3') and Clal-containing lower-strand oligonucleotide 1395C (5'-TCT TAT CCA TGT GGT ATT GAA AGA GCA-3'). This retained the original amino acid sequence of NL4-3 while creating a unique PsiAI site at 54 bases upstream from the start of the V1 region. A similar PCR amplification was done to generate a clone containing V1 sequences from clone JCR-CSF (27). An EcoRI-to-PsiAI fragment with upstream pol and env sequences from NL4-3 obtained by PCR amplification with oligonucleotides 5729 (5'–ACC CAT AAA AAG AAT TCT GC-3') and 1405C (5'–CTT TAG ACT TTG GTC CCA TAA A-3') was inserted into these plasmids to replace sequences removed earlier in clone 46-30. To create clones 21-85 (NNB) and 150-21 (CBB), the above clones were then digested with Clal and Stul and used for insertion of the Ba-L V2 region sequences from position 9 to the Stul site located nine residues downstream from the end of V2, generated by PCR amplification of 81A with the Clal-containing upper-strand oligonucleotide 1406 (5'–ACC ACA TGA ATA AGA GAT ATG TGT GAC CAA AGA GCA-3') and Stul-containing lower-strand oligonucleotide 6833C (43). V2 sequences from NL4-3 were also amplified with these same oligonucleotides and inserted to create clone 20-36 (NNB). Clone 20-36 and 49-5 were both designed NNB, but they differed at position 6 of the V2 region (Fig. 1). DNA fragments were generated for various chimeric V2 regions containing sequences from either NL4-3 or Ba-L by annealing a complex of three upper-strand oligonucleotides 1393 (5'–ACC CAT AAA AAG AAT TCT GC-3') and 1405C (5'–CTT TAG ACT TTG GTC CCA TAA A-3') was inserted into these plasmids to replace sequences removed earlier in clone 46-30. To create clones 20-36 (NNB) and 49-5 (NNB) were cloned into these plasmids to replace sequences removed earlier in clones 20-36 (NNB) and 49-5 (NNB) are identical to clone 21-85 (NNB) in the V2 amino acids upstream from the Clal site, while these clones both differ from clones 49-5 (NNB) at V2 residue 6. Therefore, clones 20-36 and 21-85 were compared to eliminate a role for V2 residue 6 variations. PBMc and was expressed as TCID 50 values (10). The titers in JC37 and PBMc assays were in close agreement.

Virusesentry experiments. To measure the efficiency of virus entry following infection of macrophages or the various HeLa-derived cell lines, total cellular DNA, including reverse-transcribed viral DNA, was isolated and quantified by real-time PCR analysis. Briefly, before infection, virus stocks were treated with DNase (Gibco) at a concentration of 200 ng/ml in 10 mM MgCl2 for 30 min at 37°C just prior to use. For negative controls in the entry assay, aliquots of the DNase-treated virus stocks were heated to 56°C for 30 min prior to infection. HeLa (CD4+ and CCR5+) cells, maintained in a rapidly growing state, were plated at a density of 2.5 × 106 cells per well of a 48-well tissue culture plate in 0.8 ml of high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum. The following day, the medium was removed, and the cells were treated for 30 min at 37°C with 0.2 ml of DEAE-dextran at a concentration of 8 μg/ml in DMEM without serum. This solution was then removed, and the cells were infected for 2 to 3 h with 0.1 ml of two serial 10-fold dilutions of virus stock. At the end of this period, 0.8 ml of DMEM with 10% fetal calf serum was added. Twelve hours later, cells were rinsed with phosphate-buffered saline (PBS). Total cellular DNA was prepared with the DNeasy Tissue kit (Qiagen Catalog number 69504). Briefly, each well received 0.2 ml of PBS, 20 μl of protease K solution, and 0.2 ml of lysis buffer (buffer AL). The tissue culture tray was washed with Parafilm, wrapped with Saran Wrap, and floated in a water bath at 70°C for 30 min. Subsequent DNA purification was accomplished according to the manufacture’s instructions. The DNA was eluted from the column with a single elution volume of 0.1 ml. The eluted DNA was used directly for real-time PCR analysis. After isolation, total cell DNA was stored at 4°C until real-time PCR analysis was conducted.

In an effort to demonstrate that the real-time PCR signal measured from total cell DNA harvested at 18 h postinfection was an accurate reflection of only the quantity of virus that entered and not an indicator of initial entry plus additional rounds of infection, preliminary experiments were conducted at multiple time points surrounding the 18-h postinfection time point. These preliminary experiments were conducted in the presence and absence of the protease inhibitor, ritonavir, which limits infection to a single round, since newly generated virus is not infectious in the absence of protease activity. No difference was noted upon comparison of the real-time PCR data in the presence and absence of ritonavir; thus, the 18-h time point was sufficiently early enough in the life cycle of the virus that only levels of initial infection would be measured (data not shown).

For entry experiments in monocyte-derived macrophages, elutriated human macrophages (Advaccine Biotechnologies Incorporated, catalog number 07-210-001) were plated at a density of 5 × 105 cells per well of a 48-well tissue culture plate. The cells were maintained in 0.8 ml of macrophage medium (DMEM, 4 mM t-glutamine, 20% fetal calf serum, and 10% human AB+ serum [Advaccine Biotechnologies Incorporated, catalog number P2-201-100]). On day 8 after plating, the medium was removed, and the macrophages were treated for 30 min
at 37°C with 0.2 ml of DEAE-dextran at a concentration of 8 μg/ml in DMEM without serum. This solution was then removed, and the macrophages were infected for 2 to 3 h with 0.1 ml of the same dilutions of virus stock as were used for the control JC37 HeLa (CD4+ and CCR5+) cells. At the end of this period, 0.8 ml of macrophage medium was added. Eighteen hours later, total cellular DNA was isolated and stored at -4°C until real-time PCR analysis was conducted.

**Real-time PCR analysis.** Reaction mixtures were (each) 30 μl containing 10 μl of total cell DNA as eluted, 15 μl of 2X PCR master mix (Applied Biosystems, catalog number 4304437), 41F primer (5'-GGCTAACTAGGAAACCTCGT C-3') at 350 nM, 118R primer (5'-CAACAGACGGGCACACACTACT-3') at concentrations from 25 to 1,000 pg/ml.

RESULTS

Influence of envelope V1 and V2 sequences on infection of macrophages. Our previous results indicated that sequences in the V1/V2 region of the HIV-1 envelope gene influence the ability of the virus to spread after infection of macrophages in vitro. To determine whether V1 or V2 or both regions were involved in this effect, recombinant chimeric infectious HIV-1 clones were generated in which the V1, V2, and V3 sequences from the macrophage-tropic strain Ba-L were inserted in various combinations in place of the original LAI envelope sequences in the infectious clone pNL4-3-10-17 derived from NL4-3. In addition to the previously described clones 81A (BBB) and 49-5 (NNB) (43), we generated clone 21-85 containing the V1 region of NL4-3 and the V2 and V3 regions of Ba-L (NNB) for the present study. We also generated a second NNB clone, 20-36, containing the V1 and V2 sequences of NL4-3 plus the V3 of Ba-L to eliminate a variability in residue 6 of V2 upstream of the Clal site in clone 49-5 (Fig. 1).

Cultures of PBMC and macrophages were infected with virus generated from the group of chimeric HIV-1 clones that contained V1 and V2 regions from NL4-3 at high and low inputs of virus per culture, and replication was measured by p24 capture enzyme-linked immunosorbent assay (Fig. 2).

Each chimeric virus grew well in the PBMC cultures, reaching high p24 levels after 10 days. After the infection of macrophages with high virus input, BBB and NBB clones grew rapidly. In contrast, NNB clones grew slowly. NNB clones reached a plateau of p24 in supernatant which was 100-fold lower than that attained by the other clones (Fig. 2). Similar results were seen for the chimeric viruses after infection with the lower input. A minor delay in BBB replication at the lower input was observed in this single experiment; however, BBB and NNB clones both spread effectively to high levels by day 20 in this and three other independent experiments. Thus, the V2 region of Ba-L appeared to be required for high-level infection and spread in macrophage cultures. On the other hand, the V1 region exerted only very limited effects when combined with V2. These effects were specific for macrophages, as there was no difference seen in the infection of PBMC by these same viruses.

Similar experiments were conducted with chimeric clones, CBB and CCB, which contained V1 and V2 regions from JR-CSF (27) instead of NL4-3 (Fig. 2). While CCB and CBB grew to similar levels in PBMC and in macrophages, CCB grew slowly compared with CBB and BBB. Hence, similar macro-

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Phage-specific effects were observed in both NL4-3 and JR-CSF chimeras. We were unable to test the effect of the Ba-L V1 without the Ba-L V2 because multiple BNB and BCB clones were not viable.

NNB clones produce infectious virus in macrophage cultures. Since the viral stocks used in the above experiments and our previous studies were made with PBMC or JC37 HeLa (CD4+ and CCR5+) cultures, one possible explanation for the lack of spread of NNB viruses in macrophage cultures might be a defect in virus output in macrophages, leading to lack of production of infectious virus after the initial infection. To test this possibility, supernatants from infected macrophages were tested for infectivity. In these experiments, NNB virus was detectable at low levels in the supernatants of the infected macrophage cultures (Table 1). Using supernatant fluids from macrophage cultures infected by BBB clones, higher levels of infectious virus were detectable. Thus, although NNB virus titers produced by macrophage cultures were lower than those of BBB viruses, this difference appeared to reflect the difference in the percent of macrophages infected by these clones, as there did not appear to be an overall inability of macrophages to release infectious NNB virus (Table 1).

NNB clones have reduced entry in macrophages. Another explanation of the lower level of infection of macrophage cultures by NNB viruses might be a defect in viral entry. To examine this possibility, macrophage and JC37 HeLa (CD4+...
and CCR5$^+$) cultures were analyzed in a viral entry assay with various HIV-1 clones. JC37 cells were utilized as the permissive positive control cell line in these entry studies to alleviate the variability associated with individual PBMC preparations. Viral entry was measured by quantitative real-time PCR analysis of reverse-transcribed viral DNA from cells 18 h after virus infection. In these studies, NNB clones showed a consistent 10-fold decrease in the entry of macrophages compared to BBB clones, whereas NBB clones showed only a 2-fold decrease (Fig. 3). This result was observed at two input levels differing by 10 fold, and appeared to explain, in part, the lower levels of infection and spread by NNB viruses in macrophages seen above.

To determine which amino acid residues in V2 might account for these differences in entry, a series of mutant V2 clones were generated and tested. A total of 6 residues out of the 40 in V2 differed in the NBB versus NNB clones used for this study (Fig. 4). In the viral entry studies, no single amino acid position appeared to completely control entry; however, this study (Fig. 4). In the viral entry studies, no single amino acid position appeared to completely control entry; however, this study (Fig. 4).

Entry of NNB clones is influenced by target cell surface CD4 concentration. While PBMC have been shown to express $\sim 10^5$ CD4 molecules per cell (28), macrophages express CD4 at relatively low or undetectable levels (15, 20, 24, 29, 44). To examine whether low cell surface concentrations of CD4 on macrophages could be responsible for the distinct entry phenotypes of the clones described above, we utilized two panels of HeLa cell lines that expressed differing levels of CD4 (35).

When cells with high CD4 levels were tested (J37), viral entry was similar for BBB, NBB, and NNB clones; thus, the differences in the V1 and V2 regions had no effect (Fig. 5). In contrast, when CD4 levels were 10- to 40-fold lower (cell lines RC49 and RC30), the pattern of entry was similar to that observed in macrophages, as BBB and NBB viruses entered at a high level, whereas NNB viruses entered poorly (Fig. 5). These results were similar whether CCR5 cell surface expression was high (RC49) or low (RC30). Since differentiated macrophages have low CD4 levels, these results suggested that the reduced macrophage entry by HIV-1 clones containing V2 sequences from NL4-3 was associated with the relatively low levels of CD4 expressed on these cells.

HIV-1 entry and spread is modulated by cell surface receptor-coreceptor concentrations and V1/V2 loop sequences. To study the role of various CCR5 concentrations in the context of both high and low CD4 expression, several additional HeLa cell lines were analyzed for viral entry by the three types of HIV-1 clones, BBB, NBB, and NNB. These results together with those shown in Fig. 5 are presented as a function of CCR5 concentration in Fig. 6. In the JC series of cell lines where CD4 expression was high, all three types of viruses were capable of efficient entry over a wide range of CCR5 expression levels. Only in JC10 cells, which expressed the lowest CCR5 levels, was a significant decrease in entry observed (Fig. 6). No entry was seen in the original CCR5-negative cell clone (HI-J), confirming that CCR5 expression was required for entry by these viruses. The slight decrease in entry using JC48 and JC24 cells appeared to be due to the loss of some of these cells from the monolayer during extensive fusion at the time of infection. In contrast to the results with JC lines, CCR5 expression level was critical in the RC lines, which expressed a 40-fold-lower CD4 level, and entry was reduced concurrently with CCR5 expression. Again, no entry was seen when CCR5 was not present (clone HI-R). In the RC cell lines, BBB virus entered at a level slightly better than NBB, and NBB in turn entered better than NNB (Fig. 6). Together these data suggest that limiting quantities of cell surface CD4 or CCR5 had dramatic effects on the entry phase of viral infection by certain HIV-1 clones, depending mostly on their V2 envelope sequences; however, V1 also

### TABLE 1. Detection of infectivity in supernatant of HIV-1-infected macrophages

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Infected macrophages</th>
<th>PBMC Titer</th>
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<tbody>
<tr>
<td>81A (BBB)</td>
<td>50</td>
<td>$9 \times 10^2$</td>
</tr>
<tr>
<td>81A (BBB)</td>
<td>25</td>
<td>$2 \times 10^2$</td>
</tr>
<tr>
<td>49-5 (NNB)</td>
<td>5</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>49-5 (NNB)</td>
<td>2</td>
<td>$1 \times 10^3$</td>
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* Macrophage cultures were infected 7 days previously with $2 \times 10^6$ TCID$_{50}$ of HIV-1 from infected PBMC; medium was changed on days 1 and 4, and supernatant fluid was collected on day 7 for analysis of infectious virus by endpoint titration on PBMC. Titers are TCID$_{50}$ values per 0.1 ml. The percent infected cells was determined by indirect immunostaining of cells with anti-p24 monoclonal antibody, 183-H12-5C, as described in Materials and Methods.
played a detectable role in combination with V2, as indicated in small differences observed between BBB and NBB clones in these experiments.

**Influence of CD4 and CCR5 concentrations on viral fusogenicity and spread.** In our previous studies, we noted that BBB clones, which spread extensively in macrophages, also induced a high level of cell fusion during infection, in contrast to NNB clones which spread poorly and failed to fuse macrophages (43). Therefore, we were also interested in studying the effects that cell surface receptor concentrations and viral V1 and V2 sequences might have on fusogenicity and spread of virus in the JC and RC HeLa cell lines. Following infection of these lines, virus was allowed to enter and spread throughout the cultures for a period of 3 days, at which time the cultures were fixed and stained for viral p24 antigen.

When CD4 concentrations were high (JC cell lines) and CCR5 levels were constant, BBB, NBB, and NNB viral clones were similar in levels of both entry and fusion, but the extent of cell fusion induced by these viruses was greatly influenced by CCR5 concentrations (Fig. 7). On JC10 cells, which have the lowest CCR5 levels, virus antigen-positive foci were found in small clusters of two to five cells with minimal fusion, whereas on JC37, antigen-positive foci were multinucleated giant cells with 10 to 20 nuclei each; on JC24 cells, with the highest CCR5 levels, viral foci were even larger than those seen with JC37 cells (Fig. 7).

Results were quite different on cells expressing lower CD4 levels. For example, in RC30 cells expressing low CD4 and low CCR5 levels, small foci with minimal fusion and spread were seen with each of the viral clones (Fig. 7). However, in RC49 cells (low CD4 and high CCR5 levels), extensive fusion with large multinucleated giant cells was induced by the BBB virus, smaller foci with fusion were induced by the NBB virus, and tiny foci with minimal fusion were induced by the NNB virus (Fig. 7). Thus, in both entry and fusion-spread assays, the RC49 cell line appeared to act similarly to macrophages infected by these same clones.

**DISCUSSION**

In our previous studies, the V1 and V2 hypervariable domains of the HIV-1 envelope surface glycoprotein influenced the replication efficiency of macrophage-tropic HIV-1 by affecting virus spread in macrophage cultures but not in PBMC cultures (43). In the present experiments, V2 residues had the most influence on spread and replication in macrophages, while the V1 residues exerted a lesser effect. Measurement of NNB virus production by macrophages indicated that the infectious virus was produced in a quantity proportional to the number of infected cells. Therefore, lack of NNB virus production by macrophages did not appear to explain the low levels of replication and spread seen in macrophages infected with NNB clones. In contrast, infectious entry into macrophages was 5- to 10-fold higher for NBB and BBB viruses, respectively, than for NNB viruses (Fig. 3). This effect was macrophage specific and was not seen in JC37 HeLa (CD4+ and CCR5+) cells (Fig. 5). Therefore, this defect in virus entry...
appeared to correlate well with the poor replication and spread of NNB viruses in macrophages. While all HIV-1 clones tested were able to enter HeLa-CD4 and CCR5 cells expressing high levels of CD4, our experiments conducted in HeLa cells expressing lower cell surface CD4 concentrations provide a possible explanation for how viruses containing NL4-3 V1 and V2 hypervariable regions distinguish macrophages from PBMC. These HeLa cells were not efficiently entered by viruses containing NL4-3 V1 and V2 hypervariable regions (NNB) (Fig. 5), whereas macrophages efficiently entered viruses with a negatively charged glutamic acid residue at V2 position 22 instead of a positively charged lysine residue from the NL4-3 clone (Fig. 4). However, clone BN8, which had only this substitution had a low entry level, and one clone (BN2), lacking glutamic acid at position 22, had intermediate levels of entry. These results indicate that this single amino acid substitution is neither necessary nor sufficient for a high entry level, and other structural features must also be important. For example, V2 contains three N-linked glycosylation sites that modulate the interaction of HIV-1 envelope with CD4 and CCR5 to specifically influence macrophage infection but not PBMC infection (30). In our clones, the glycosylation site at residue 32 in the V2 of Ba-L was relocated to residue 30 in the V2 of NL4-3. Perhaps these differences in glycosylation sites in the V2 region also influence viral entry level. Since no single amino acid residue or distinct region of V2 appeared to completely control entry, it is likely that overall V2 conformation is an important factor in the effects observed.

While the HeLa cell entry data suggested a definitive role for CD4 concentration in modulation of macrophage entry, the precise mechanism of V1 and V2 involvement is not known. Since no V1 or V2 residues have been shown to directly interact with CD4, it is unlikely that a direct V1/V2–CD4 interaction could account for the observed CD4-dependent effects of V1/V2. On the other hand, two distinct mechanistic possibilities could explain how CD4 concentrations could differentially modulate entry of viruses containing different V1/V2 loops. The conformation of envelopes containing certain V1 and V2 residues and/or glycosylation patterns could indirectly influence CD4 binding by other regions of envelope. Alternatively, when target cell surface CD4 is limiting, envelope conformational changes that are necessary for productive interactions with CCR5 might be more efficiently attained when certain V1
FIG. 6. Virus entry into HeLa cells as a function of receptor and coreceptor concentrations. Three chimeric viruses (81A [BBB], 21-85 [NBB], and 20-36 [NNB]) were used to infect HeLa cultures expressing various levels of surface CD4 and CCR5. Solid bars indicate percent relative entry into RC lines (low CD4) while hatched bars indicate entry into JC lines (high CD4). Percent relative entry was calculated utilizing real-time PCR analysis of reverse-transcribed HIV DNA contained within the infected HeLa cells. Levels of entry are normalized to levels of entry for each clone in JC37. The numerical designation of each cloned cell line as well as the relative levels of CD4 and CCR5 expressed on the surface of each line are indicated to the left of the histograms. Error bars represent the standard errors of the mean. No entry was seen in HI-J and HI-R HeLa-CD4 lines, which did not express CCR5. The data for each point was collected in four independent experiments in which each data point was assayed in duplicate.

FIG. 7. Immunohistochemical detection of HIV-1-infected foci in HeLa cultures. HeLa cell cultures were infected with a chimeric virus and on day 3 postinfection were fixed and stained for p24 by an indirect immunoperoxidase technique. The HeLa cell line utilized in each focus assay along with approximations of the relative concentrations of CD4 and CCR5 expressed on the surface of each line are indicated to the left of each row of micrographs. The chimeric virus clone used for infection is indicated above each column of micrographs. No foci were seen in HI-J and HI-R HeLa-CD4 lines which did not express CCR5 (data not shown).
and V2 sequences and/or glycosylation patterns are present. In either case, viruses containing Ba-L V2 sequences might require fewer CD4 interactions for entry than do NL4-3 V2 viruses.

The current model of HIV entry involves two sequential conformational changes in gp120 prior to activating the actual fusion machinery in gp41 (for reviews, see references 5 and 34). The first conformational shift occurs as a consequence of the interaction of CD4 with envelope and results in the creation, stabilization, or exposure of a coreceptor (i.e., CCR5) binding site on gp120. Once the envelope binds to the coreceptor, the resulting conformational change induces activation of the fusion domains in gp41 that ultimately mediate fusion and/or entry. One role of V1/V2 in certain HIV isolates may be to limit the exposure of certain envelope regions involved in coreceptor binding until sufficient CD4-envelope interactions have occurred as a way of hiding the coreceptor binding site from antibody binding (4, 7, 26, 32, 38, 42, 47, 48). In this model, part of the envelope coreceptor binding site is occluded by a V1/V2 arm that is displaced upon CD4-envelope interaction exposing the coreceptor binding site.

In agreement with the above hypothesis, our entry and fusion spread data (Fig. 6 and 7) suggest that certain V2 region sequences (e.g., NL4-3) can impede HIV-1 entry into cells expressing limited surface CD4 concentrations. When the NL4-3 V2 loop was present (NNB viruses) and CD4 concentrations were low, entry and fusion spread were severely diminished. However, if CD4 expression was increased, this block was removed, entry was rescued, and fusogenicity became a direct function of CCR5 concentration. On the other hand, if the Ba-L V2 loop was present (BBB and NBB viruses), both entry and fusogenicity were still dependent on CCR5 concentrations, but no difference was seen in the two CD4 concentrations tested. Thus, the entry block imparted by NL4-3 V2 sequences could be removed either by increasing target cell surface CD4 concentrations or by substitution of nonblocking V2 sequences (e.g., Ba-L). While our work does not describe the precise molecular interactions involved in HIV entry, these studies are in agreement with the hypothesis of Sodroski and colleagues suggesting that certain V1 and V2 loops (e.g., NL4-3 V1/V2) might act to block CCR5 access to its envelope binding site until CD4 binds (32, 42, 47, 48). It is possible that certain V1/V2 sequences (e.g., Ba-L V1/V2) could impart increased structural flexibility to the V1/V2 loop, resulting in a decrease in the energy barrier required for the conformational rearrangements necessary for exposure of the CCR5 binding site. Hence, in the case of Ba-L V1/V2 viruses, fewer CD4-envelope interactions would be necessary for entry compared with the more structurally constrained V1/V2 of NL4-3 viruses.

Since macrophages and microglia have been shown to express limited cell surface CD4, the above findings may have implications for cell tropism and selection of viruses in tissues such as lung and brain, where macrophages or microglia are numerous. In such tissues, one would predict selection of HIV clones with the V1 and V2 sequences associated with efficient and rapid spread in macrophages. In fact, this result was observed in our previous study of HIV-1 envelope sequences isolated directly from the brains of infected individuals (36). Similar findings have been reported by other groups (19, 33).

Possibly these envelope sequences not only facilitate increased replicative capacity for microglia but also mediate additional pathogenic potential for brain tissue, leading to the syndrome of HIV-associated dementia or other central nervous system manifestations of HIV infection (19, 33). Furthermore, the selective pressures present under conditions of limiting CD4 might eventually favor CD4-independent viruses, as has been suggested in both studies conducted with HIV and simian immunodeficiency virus (6, 37).

At early times in the course of infection, macrophage-tropic viruses are usually present. In one study, macrophage-tropic viruses isolated immediately after seroconversion had a slow-replication phenotype, whereas at later time points more rapidly replicating macrophage-tropic viruses were isolated (13). While the authors demonstrated that both early and late isolates could infect macrophages, the efficiency of entry was not directly assayed. Possibly the slow-replicating strains found shortly after transmission are similar to the NNB viruses studied here. However, it remains unclear what advantage these slow-spreading clones might have in the complex set of events that occur during transmission to a new host. In any case, these viruses would be likely to be important targets for future vaccine development.

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HIV-1 INFECTION OF MACROPHAGES


