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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. By 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.

Infection of cells by human immunodeficiency virus (HIV) begins with the sequential interaction of the viral envelope glycoproteins with cellular receptor molecules including CD4 plus one of several chemokine receptors. While more than a dozen chemokine receptors are known to mediate HIV entry, the two that are most commonly utilized by HIV isolates are CXCR4 and CCR5. CXCR4 HIV variants (T-cell tropic) typically arise late in the disease course and utilize CXCR4 coreceptors that are expressed on T cells (17), while CCR5 variants (macrophage tropic) are typically the transmitted form and enter cells using CCR5 coreceptors that are expressed on macrophages and T cells (2, 14, 16). The primary determinants necessary for CCR5 or CXCR4 coreceptor usage have been previously mapped to the V3 region of the envelope. However, this domain is not sufficient for high levels of virus infection in macrophages, as previous experiments have shown that both nonenvelope and envelope regions other than V3 can alter macrophage infection (3, 23, 25, 31, 45).

Previously, sequences within the V1 and V2 regions of HIV envelope have been shown to influence various aspects of macrophage infection. For example, V2 can modulate the influence of V3 on macrophage tropism and also influences envelope interactions with soluble CD4 (25, 41). In addition, V1 and V2 changes may be required for the efficient production of virus in macrophages (40, 46). In our previous studies, differences in V1 and V2 envelope sequences were shown to be responsible for alterations in efficiency of HIV-1 spread in monocyte-derived macrophages (43). This effect was specific for macrophages in that the influence of V1 and V2 regions was not seen during infection of lymphocytes. Other V1 and V2 region differences were associated with variations in the extent of macrophage tropism and the ability to infect cells expressing low CD4 levels (33) and with the ability to induce apoptosis in neuronal and glial cells (19). In HIV-1 isolates adapted to growth in microglia, V1 and V2 region differences were associated with altered fusogenicity of microglia, although additional substitutions in nonenvelope regions were required for maximal replication in these cells (39). Additionally, studies of simian immunodeficiency virus–HIV-1 chimeric virus suggest that specific V2 region alterations are responsible for in vivo evolution of macrophage tropism from an exclusively T-cell-tropic simian immunodeficiency virus–HIV-1 chimeric virus in a rhesus monkey model (21, 22). In summary, while the V1/V2 region influences macrophage tropism, microglial tropism, and neurovirulence, the molecular mechanisms behind these effects have not been elucidated.

In the present study, use of new infectious recombinant and mutant HIV-1 molecular clones demonstrated that the main sequences responsible for efficient virus spread in macrophages were located in the V2 region. The mechanism of this effect on HIV-1 spread in macrophages appeared to involve an effect on virus entry into macrophages. In an effort to mimic

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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 BBB) 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 StuI 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 PshAI, and a BamHI linker was inserted to remove this unique PshAI site. An oligonucleotide polylinker with sites EcoRI-SalI-PshAI-ClaI-StuI was inserted between the unique EcoRI and StuI sites to generate clone 46-30. Next, the NL4-3 V1 region plus sequences encoding 18 upstream residues plus 8 residues of V2 from NL4-3 was inserted between PshAI and ClaI with a PCR fragment obtained by amplifying pNL4-3-10-17 (8) with PshAI-containing upper-strand oligonucleotide 1393 (5′-TGG GAC CAA AGT CTA AAG CCA TGT G-3′) and ClaI-containing lower-strand oligonucleotide 1395C (5′-TCT TAT CGA TGT GGT GAT ATT GAA AGA GCA-3′). This retained the original amino acid sequence of NL4-3 while creating a unique PshAI site 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 JR-CSF (27). An EcoRI-to-PshAI fragment with upstream *pol* and *env* sequences from NL4-3 obtained by PCR amplification with oligonucleotides 5729 (5′-AGC CAT AAT 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 (NBB) and 150-21 (CBB), the above clones were then digested with ClaI and StuI and used for insertion of the Ba-L V2 region sequences from position 9 to the StuI site located nine residues downstream from the end of V2, generated by PCR amplification of 81A with the ClaI-containing upper-strand oligonucleotide 1406 (5′-ACC ACA TCG ATA AGA GAT AAG GTG CAG A-3′) and StuI-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). Clones 20-36 and 49-5 were both designated 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 and three lower-strand oligonucleotides as described previously (12), and these V2 sequences were inserted as described above. Clone 146-7 (CCB) was generated by insertion of an EcoRI to a PshAI PCR fragment obtained by amplifying pNL4-3-10-7 with oligonucleotides 5729 and 1405C into clone 46-30 to generate clone 67-11. A PCR fragment obtained by amplification of JR-CSF with PshAI-containing upper-strand oligonucleotide 1393 and StuI-containing lower-strand oligonucleotide 6833C was then inserted into the PshAI and StuI sites of 67-11 to create clone 146-7 (CCB).

Virus stocks. Plasmid DNA from molecular clones was used to transfect JC37 CD4- and CCR5-positive ($CD4^+$ and $CCR5^+$) HeLa cells (35), and these cells were cocultivated with phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) to produce virus stocks as described previously (8, 9). Alternatively, JC37 cell supernatants harvested at 48 to 72 h posttransfection were used to infect fresh JC37 cell cultures which were approximately 25% confluent. Two days later, when the cells were nearing confluence and the cultures contained large numbers of multinucleated cells, the medium was changed. Twenty-four hours later, the supernatant was harvested, centrifuged, aliquoted, and frozen at −70°C. This was done for 2 consecutive days. Virus stocks from JC37 cells regularly produced higher titers than those from PBMC. Stocks from both sources behaved identically in all assays tested. Virus titers were calculated and expressed as focus-forming units (FFU) or the 50% tissue culture infectious dose ($TCID_{50}$). Briefly, HeLa JC37 cultures (35) were infected and foci were stained by immunohistochemistry (as detailed below). These results were expressed as the number of FFU per milliliter. In other experiments, virus infectivity was measured as previously described by endpoint dilution on PBMC and was expressed as $TCID_{50}$ values (10). The titers in JC37 and PBMC assays were in close agreement.

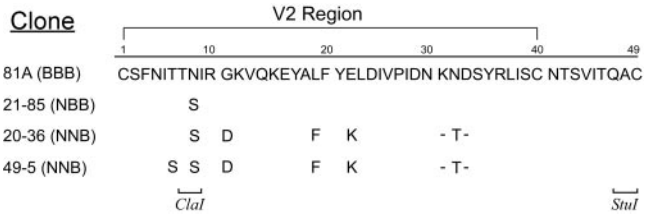

FIG. 1. Comparison of envelope amino acid sequences from the V2 regions of four chimeric infectious molecular HIV-1 clones. Recombinant clones were generated by the insertion of V1, V2, and V3 from the macrophage-tropic strain Ba-L in place of the original LAI envelope sequences of the infectious clone pNL4-3-10-17 derived from NL4-3. The names of each clone are indicated to the left of each sequence, followed by letters in parentheses that indicate whether the V1, V2, or V3 regions were derived from NL4-3 (N) or Ba-L (B), respectively. Amino acid residues are numbered starting from the first cysteine residue of the V2 hypervariable domain. Dashes indicate deleted residues. Relative locations of ClaI and StuI restriction endonuclease sites utilized in cloning reactions are indicated. Clone 20-36 (NNB) is identical to clone 21-85 (NBB) in the V2 amino acids upstream of the ClaI site, while these clones both differ from clone 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.

Virus entry 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 $MgCl_2$ 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 2.5×10^4 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. Eighteen 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 proteinase K solution, and 0.2 ml of lysis buffer (buffer AL). The tissue culture tray was sealed with Parafilm, wrapped with Saran Wrap, and floated in a water bath at 70°C for 30 min. Subsequent DNA purification was completed according to the manufacturer'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 monocytes (Advance Biotechnologies Incorporated, catalog number 07-210-001) were plated at a density of 5×10^5 cells per well of a 48-well tissue culture plate. The cells were maintained in 0.8 ml of macrophage medium (DMEM, 4 mM L-glutamine, 20% fetal calf serum, and 10% human AB+ serum [Advance 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 2× PCR master mix (Applied Biosystems, catalog number 4304437), 41F primer (5′-GGCTAACTAGGGAACCCACTG C-3′) at 350 nM, 118R primer (5′-CAACAGACGGGCACACACTACT-3′) at 350 nM, and U5 probe (6-carboxyfluorescein-AAGCCTCAATAAAGCTTGCC TTGAGTGCTC-6-carboxytetramethylrhodamine) at 180 nM. The probe and primers, specific to the HIV-1 U5 region, were designed with the Applied Biosystems software Primer Express. The 30-μl reaction mixtures were split between two wells in a 384-well plate to provide duplicate critical threshold measurements. Neither the presence of DNeasy column elution buffer nor the presence of uninfected cell DNA altered the critical threshold values (C_T) obtained for a plasmid standard. PCR thermal cycling was carried out using the default settings for the AB 7900HT Sequence Detection system (10-min denaturation at 95°C, followed by 40 cycles, each consisting of 15 s of denaturation at 95°C and 1 min of annealing and extension at 60°C). Data shown in Fig. 3 and 4 were calculated by obtaining the C_T value (cycle where the PCR amplification initiated the linear phase) for each sample. ΔC_T was calculated for each virus by subtracting the C_T value for entry in JC37 cells from the C_T value for entry of the same sample in macrophages, and the ΔC_T values were normalized to entry by clone 81A by subtracting the 81A value from each to obtain a $\Delta\Delta C_T$ value. The percent entry for each clone was calculated by the following formula: percent entry relative to clone 81A = $100(2^{-\Delta\Delta CT})$. For the results shown in Fig. 5, data was calculated similarly, except ΔC_T represented the C_T value for entry in JC24 cells subtracted from the C_T value for entry of the same virus stock in the indicated HeLa cell line. Calculations for the results shown in for Fig. 6 were similar, except ΔC_T was calculated for each virus by subtracting the C_T value for entry in JC37 cells from the C_T value from entry of the same virus in the indicated HeLa cell line, and the percent entry was calculated with the above equation without 81A normalization.

Immunohistochemical detection of HIV-infected foci. Three days after infection of the HeLa (CD4$^+$ and CCR5$^+$) cells as described above, cells were stained for p24 antigen. The medium was removed, and the cells were fixed with 95% ethanol for 10 min, then rinsed twice with wash buffer (0.01 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.002 M EDTA, 1% fetal bovine serum [FBS]). The wash buffer was aspirated, and 0.15 ml of supernatant from hybridoma 183-H12-5C (AIDS Research and Reference Reagent Program [ARRRP], catalog number 1513) was added. After incubation at room temperature for 30 min, the antibody was aspirated and the cells were washed twice with wash buffer. An aliquot of 0.15 ml of peroxidase-conjugated goat anti-mouse immunoglobulin (ICN/Cappel catalog number 55556) at a 1/500 dilution in wash buffer was added. The cells were then incubated at room temperature for 30 min. After this incubation, the antibody was aspirated, and the cells were washed twice with wash buffer. Then, 0.3 ml of peroxidase substrate (0.25 ml of 2 M sodium acetate [pH 5.0], 0.5 ml of 4 mg of 3-amino-9-ethylcarbazole [Sigma catalog number A-5754]/ml in dimethylformamide [Sigma catalog number D-4254], and 5 μl of 30% H_2O_2 [Calbiochem catalog number 386790] to final volume of 10 ml with H_2O) was added, and the cells were incubated for 30 min at room temperature. The substrate was aspirated, the cells were washed with water, and the foci were counted with a Nikon SMZ-10 microscope. JC37 cells were used in the virus titrations, and the results were expressed as the number of FFU per milliliter. Alternatively, light micrographs were generated utilizing a Nikon Eclipse TE300 microscope and the associated Nikon DXM1200 digital camera.

Assay for p24. At 3- to 4-day intervals, one-half of the supernatant was removed from infected PBMC cultures and from infected macrophage cultures and frozen at −70°C. The cultures were then fed with growth medium, and incubation was continued at 37°C until the next time point. The supernatant was assayed for p24 protein with a p24 capture enzyme-linked immunosorbent assay developed in this laboratory. Briefly, the anti-p24 monoclonal antibody 183-H12-5C (ARRRP catalog number 3537) was diluted 1/800 in 0.05 M sodium carbonate buffer (pH 9.6) or in PBS (pH 7.2), and 0.1 ml was added to each well of Immulon 2 HB plates (Thermo Labsystems catalog number 3655). The plates were incubated overnight at 37°C and then washed once with PBS. Plates were blocked by incubation with 0.25 ml of PBS containing 5% FBS for 1 h at 37°C. Plates were washed twice with wash buffer (PBS containing 0.2% Tween 20). Culture supernatants were mixed with 1/10 volume of PBS containing 10% Triton X-100. Dilutions of supernatants and subsequent antibodies were made in PBS containing 5% FBS and 0.5% Triton X-100. An aliquot of 0.1 ml of the diluted culture supernatant was then added to each well, and the trays were incubated at 37°C overnight. The plates were washed four times with wash buffer, and 0.1 ml of a 1:1,000 dilution of human anti-HIV antibody (ARRRP catalog number 192), biotinylated by standard procedures, was added to each well. The plate was incubated for 1 h at 37°C and washed four times with wash buffer. An aliquot of 0.1 ml of horseradish peroxidase-conjugated streptavidin (Oncogene catalog number OR03L) at a 1/2,000 dilution was added to each well, and the trays were incubated for 30 min at 37°C. The plates were washed six times with wash buffer, 0.1 ml of peroxidase substrate (10 ml of 0.01 M sodium acetate [pH 5.0], 0.3 ml of 4 mg of tetramethylbenzidine [Calbiochem catalog number 613545]/ml in dimethyl sulfoxide, and 5 μl of 30% H_2O_2) was added, and the plates were incubated at room temperature until the optical density at 630 nm of the most concentrated standard (1,000 pg/ml) was 1.2. The reaction was stopped with 0.05 ml of 2 M H_2SO_4, and the optical density was read at 450 nm. Values were compared to a standard curve made with p24 (ARRRP catalog number 382) 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 (NBB) 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 ClaI 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 NBB 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-

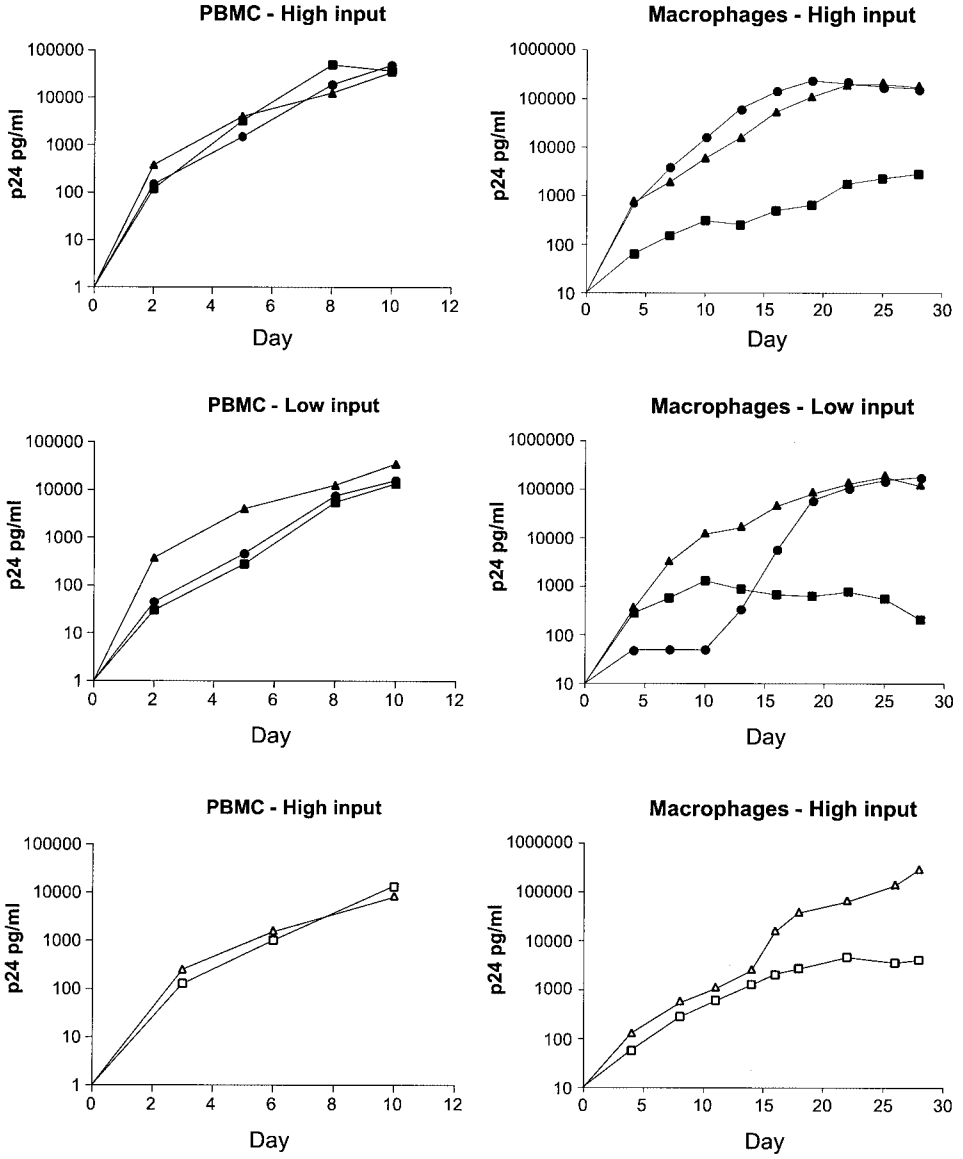

FIG. 2. Influence of HIV-1 V1 and V2 envelope sequences on kinetics of p24 production following infection of PBMC (left) and macrophage (right) cultures with either high or low inputs of virus generated from clone 81A (BBB) (●), 21-85 (NBB) (▲), 49-5 (NNB) (■), CBB (△), or CCB (□). High-input experiments were done using a 1,000 to 2,500 PBMC $TCID_{50}$ for infection, and low-input experiments were conducted using a 100 to 250 PBMC $TCID_{50}$. The V2 region of Ba-L was required for high-level infection of macrophages but not PBMC. These data are from a single experiment that was representative of results obtained in four independent experiments.

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 ($CD4^+$

TABLE 1. Detection of infectivity in supernatant of HIV-1-infected macrophages[a]

Virus	% Infected macrophages	PBMC Titer
81A (BBB)	50	9×10^2
81A (BBB)	25	2×10^2
49-5 (NNB)	5	3×10^1
49-5 (NNB)	2	1×10^1

[a] Macrophage cultures were infected 7 days previously with 2×10^3 $TCID_{50}$s 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.

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, three mutant clones (BN4, BN5, and BN1) entered cells similarly to the NBB parental clone (Fig. 4). The only common

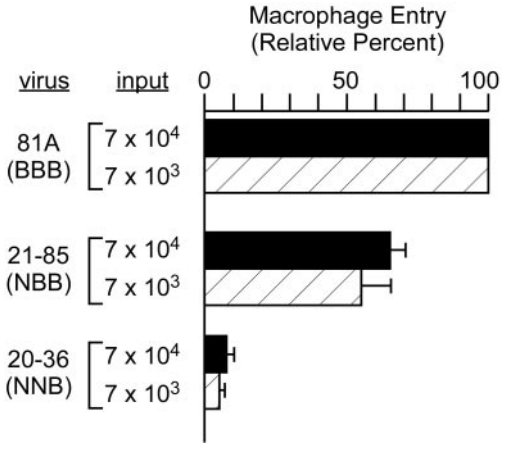

FIG. 3. Comparison of the relative abilities of chimeric HIV-1 clones to enter macrophages. The values shown are relative percent entry, normalized to clone 81A (BBB). Calculation of relative percent entry was described in Materials and Methods. Error bars represent the standard errors of the mean. The virus input represents FFU as determined by titers of the virus on JC37 HeLa (CD4+ and CCR5+) cells. The real-time PCR data for each point was collected in five independent experiments in which each data point was assayed in duplicate for two separate virus dilutions.

mutant residue in all three of these clones was glutamic acid (E) at position 22, but this change alone in mutant BN8 was not sufficient to give a high entry level. These data indicated that multiple residues in V2 are required to produce the observed changes in viral entry associated with the V2 region of the Ba-L clone.

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). The JC series expressed $\sim 4 \times 10^5$ CD4 molecules per cell, which is similar to PBMC, while the RC series expressed $\sim 10^4$ CD4 molecules per cell, which is similar to levels previously estimated on differentiated macrophages (20, 24). In addition to the two distinct levels of cell surface CD4, multiple JC and RC subclones expressed various, defined levels of CCR5 (35).

When cells with high CD4 levels were tested (JC37), 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

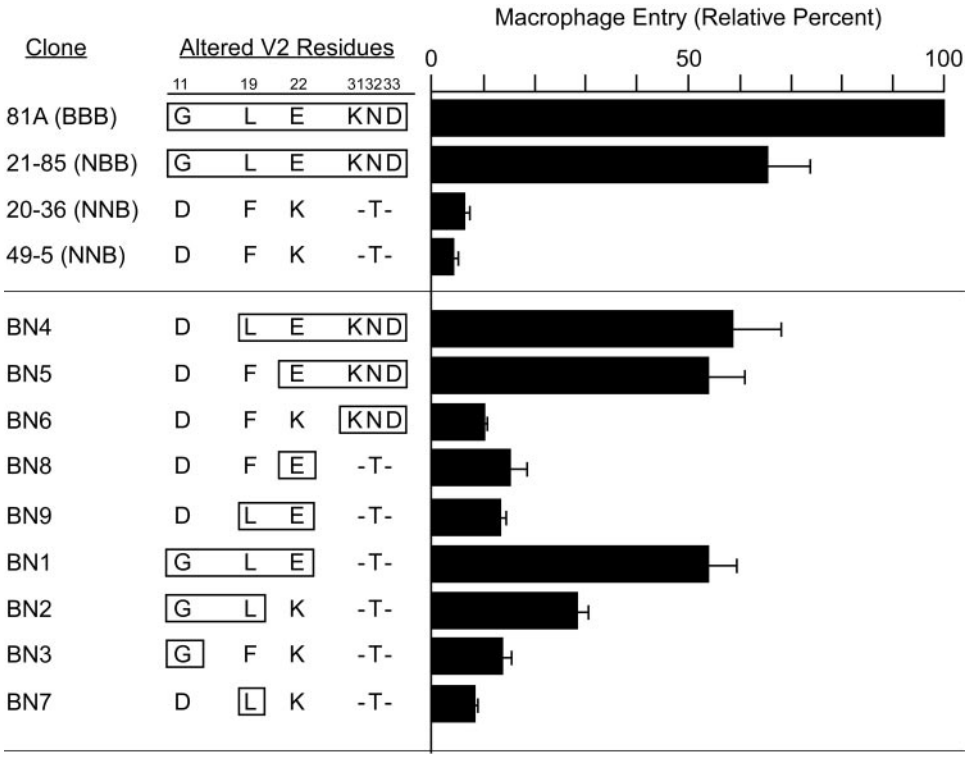

FIG. 4. Macrophage entry by parental and mutant HIV-1 clones differing in V2 residues as shown. Boxed residues are from the Ba-L clone and unboxed residues are from the NL4-3 clone. Values shown are percent entry relative to the 81A clone (100%). The real-time PCR data for each point was collected in three independent experiments in which each data point was assayed in duplicate on two separate virus dilutions.

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

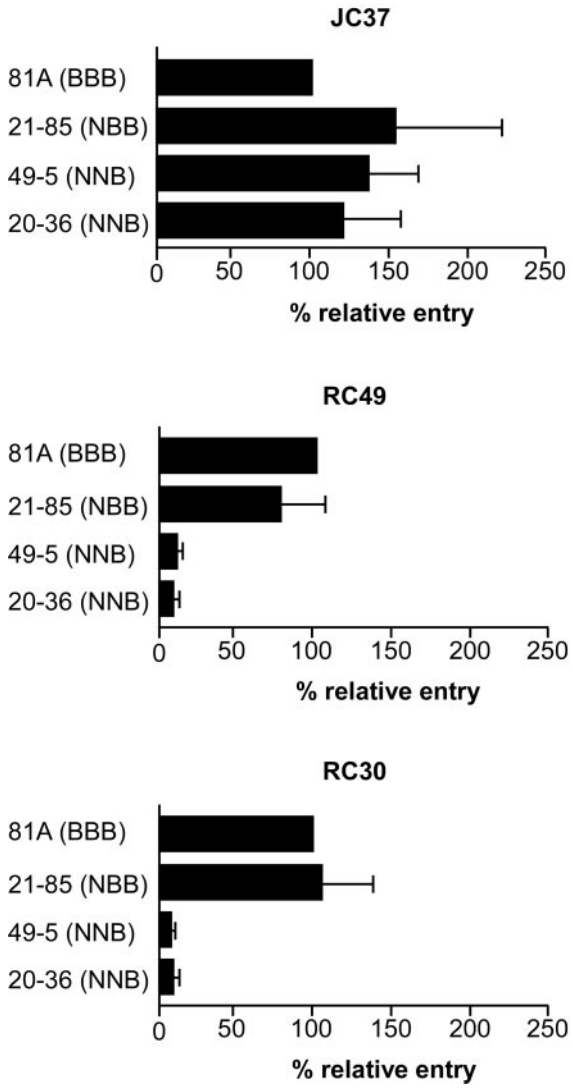

FIG. 5. Comparison of the relative abilities of different virus clones to enter HeLa cells expressing high (JC37) or low (RC49 and RC30) levels of cell surface CD4. HeLa cell cultures were infected with recombinant virus clones. The virus clone names are indicated on the left. The bars correspond to the percent relative entry normalized to 81A (BBB) entry within the same cell line. Error bars represent the standard errors of the mean. The real-time PCR data for each point was collected in four independent experiments in which each data point was assayed in duplicate.

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 and 6). Since macrophages have been shown to express low levels of cell surface CD4 concentrations (15, 24, 29, 44), a similar effect is likely to occur in macrophages. Therefore, we propose that NNB viruses distinguish macrophages from PBMC based upon cell surface CD4 concentrations. While cells that express CD4 above a threshold level (JC lines and PBMC) can be efficiently entered by viruses with NL4-3 V1 and V2 regions, cells that express CD4 below this threshold (RC lines and macrophage) are not. Our data using clones expressing V1 and V2 sequences from JR-CSF (Fig. 2) suggest that this effect might be a general phenomenon. This conclusion is supported by numerous previous reports describing HIV clones or isolates with differing abilities to infect cells expressing low CD4 levels. In some cases, this ability has correlated with the distinction between macrophage-tropic and non-macrophage-tropic viruses (4, 33), but in other cases this ability has been associated with an enhanced ability to cause apoptosis in neuronal cultures (19) or increased replication capacity in microglia (39). The results in the latter study differ from our data in that the main effect of viral envelope appeared to map to the V1 region, rather than to V2. In contrast, one previous study demonstrated that the reduced macrophage entry observed for a group of R5 primary isolates was not moderated by increased expression of CD4 (18), so CD4 level may not be the only macrophage attribute capable of influencing efficiency of HIV infection.

In an effort to map more precisely V2 residues responsible for the effects observed on virus entry and spread in macrophage cultures, we generated additional infectious recombinant HIV-1 clones that contained NL4-3–Ba-L chimeric V2 sequences. All viruses that efficiently entered macrophages had a negatively charged glutamic acid residue from the Ba-L clone 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

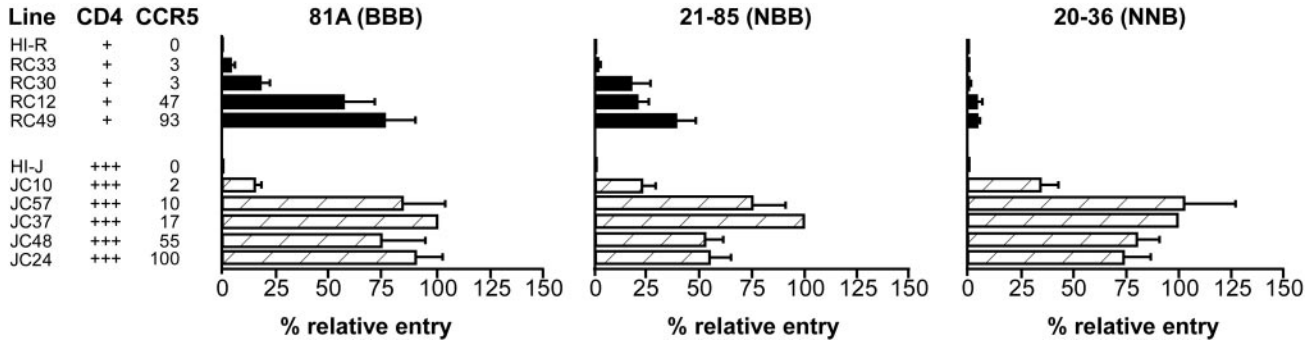

FIG. 6. Virus entry into HeLa cells as a function of receptor and coreceptor concentrations. Three chimeric viruses (81A [BBB], 21-85 [NBB], and 20-36 [NNB]) were used to infect HeLa cultures expressing various levels of surface CD4 and CCR5. Solid bars indicate percent relative entry into RC lines (low 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.

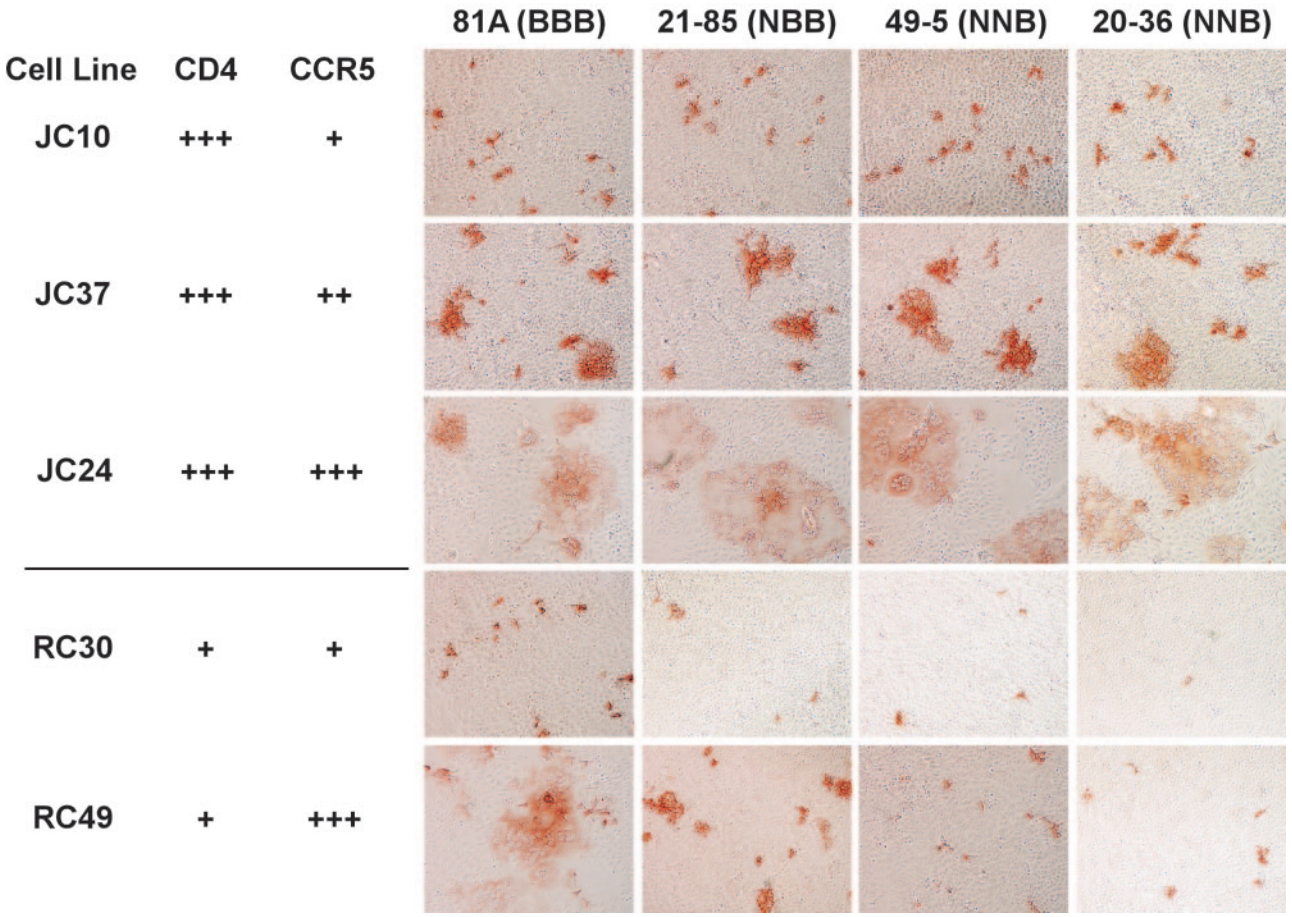

FIG. 7. Immunohistochemical detection of HIV-1-infected foci in HeLa cultures. HeLa cell cultures were infected with a chimeric virus and on day 3 postinfection were fixed and stained for p24 by an indirect immunoperoxidase technique. The HeLa cell line utilized in each focus assay along with approximations 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, 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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