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Human Immunodeficiency Virus Type 1 Tropism for Brain Microglial Cells Is Determined by a Region of the *env* Glycoprotein That Also Controls Macrophage Tropism

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Human immunodeficiency virus type 1 (HIV-1), the agent of AIDS, frequently infects the central nervous system. We inoculated adult human brain cultures with chimeric viruses containing parts of the *env* gene of a cloned primary isolate from brain tissue, HIV-1 JRFl, inserted into the cloned DNA of a T-cell-tropic strain. A chimeric virus containing the carboxy-terminal portion of HIV-1 JRFl *env* did not replicate in these brain tissue cultures, while a chimera expressing an *env*-encoded protein containing 158 amino acids of HIV-1 JRFl gp120, including the V3 loop, replicated well in brain microglial cells, as it does in blood macrophages. Infection of brain microglial cells with such a chimera was blocked by an antibody to the V3 loop of gp120. Thus, *env* determinants in the region of gp120, outside the CD4-binding site and comprising the V3 loop, are critical for efficient viral binding to and/or entry into human brain microglia.

Human immunodeficiency virus type 1 (HIV-1) not only infects T cells and monocyte-macrophages (MM) but can also invade the nervous systems of HIV-1-seropositive patients at an early stage of the disease or when AIDS has fully developed (14). Expression of the virus in the brain has been correlated with the neuropsychiatric syndrome called the AIDS dementia complex (ADC), and HIV-1 has been isolated from the brains of patients with ADC (10; reviewed in references 14, 20, and 26). In the central nervous system, HIV-1 infection can be widespread, with viral RNA and protein found predominantly in microglial cells, which sometimes form giant cells, and in perivascular infiltrates (reviewed in references 7 and 26). Microglia are cells derived from bone marrow precursors, expressing MM markers and residing in the mature brain (11, 25). To study the cellular and molecular basis of HIV-1 neurotropism, we have established an in vitro system consisting of adult human brain primary cultures which contain all known types of glial cells, including astrocytes and microglia (35). Such human brain cultures can be productively infected with MM-tropic HIV-1 strains which replicate selectively in microglial cells, causing cell fusion and death (7, 35). This observation correlates well with the neuropathological analysis of nervous tissues of patients with ADC, as well as the fact that HIV-1 brain isolates usually grow more efficiently in MM (3, 26).

The CD4 molecule which functions as the HIV-1 receptor in T lymphocytes and MM (30) is expressed in human brain-derived microglial cells and mediates viral entry into these cells (15). A region located in the carboxy-terminal portion of viral envelope glycoprotein gp120 is crucial in receptor recognition and binding to CD4 (19, 30). Recent studies have shown that another region of gp120 determines the ability of certain HIV-1 strains to enter and/or replicate in MM—as well as T cells—and this region consists of, at most, 159 amino acids (aa) which include the V3 loop, the principal neutralizing determinant of gp120 (2, 23, 27, 32, 36). Since microglial cells constitute the main site of HIV-1 replication in the brain (reviewed in references 7 and 26), we investigated whether determinants in the *env*-encoded protein determine the ability of HIV-1 to infect human brain microglia in vitro. We chose to use chimeric viruses with a portion of the gp120 of the JRFl strain (23) because this virus was isolated from the brain of a patient with ADC (17) and because after two passages in peripheral blood lymphocytes (PBLs), it replicated to high titers in cultured human microglial cells, causing a release of 70 to 100 ng of $p24^{gag}$ protein into the supernatant (7).

Differential growth of *env* chimeric viruses in human brain cultures. Surgically resected temporal lobe tissue from epileptic patients resistant to drug treatment was used to establish and grow primary cultures of a portion of grey and/or white matter outside the epileptic focus as described previously (1, 35). We routinely added to the medium 10% giant cell tumor supernatant—a mixture of lymphokines (8)—to enhance survival of the brain-derived microglial cells in our cultures. Microglial cells were identified by expression of the low-density lipoprotein receptor and astrocytes by the presence of glial fibrillary acidic protein, an intermediate filament protein, by using double and fluorescence labelling techniques (35). Cultures were inoculated after 1 to 2 weeks in vitro with chimeric viruses grown in phytohemagglutinin-stimulated PBLs (23). These recombinant viruses contain

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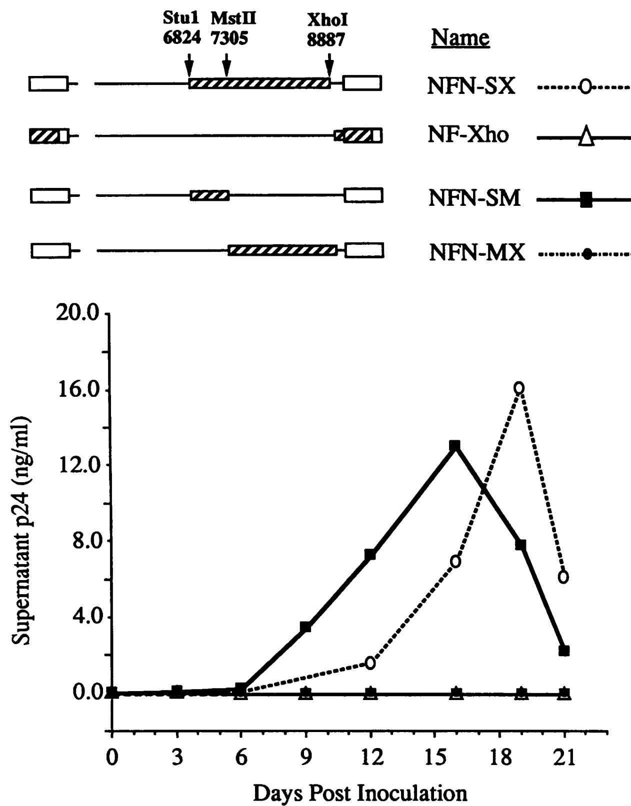


FIG. 1. Kinetics of p24^{gag} production by human brain cultures after inoculation with four different chimeric viruses. The DNA structures of these chimeras are shown at the top (for details, see reference 23). Three different restriction sites were used to insert the different portions of the *env* gene of a brain isolate clone (JRFl [▨]) into the context of a T-cell-adapted strain (NL4-3 [▭]). Base pair numbering corresponds to that of NL4-3 (22). After inoculation of primary human brain cultures, only two of the four chimeric viruses tested replicated, as assayed by release of p24 antigen into the supernatant over a 3-week period. Chimeric virus NFN-MX, which contains a 3′ region of the JRFl *env* gene does not replicate in these cultures. Each point represents the values for the pooled supernatants of three cultures of one representative experiment. Three to six experiments were performed for each viral strain, giving similar results.

part of the *env* gene of a JRFl clone inserted into the cloned DNA of T-cell-tropic strain NL-4-3 and are represented in the upper part of Fig. 1 (23). NFN-SX contains the largest portion of the *env* gene of JRFl (nucleotides 6822 to 8887 [base pair numbers refer to NL 4-3; see reference 22 for the corresponding JRFl sequences]), excluding the regions that code for the first exons of regulatory genes *tat*, *rev*, and *vpu* (23). NF-Xho contains almost exclusively the NL4-3 viral genome, except for a portion of the *nef* gene and the U3/R regulatory sequences. Given that these viral strains exhibit variable abilities to grow in different cell types, it was not possible to establish a meaningful 50% tissue culture infective dose that applied to all strains; therefore, the inocula were normalized with respect to p24^{gag} content. Human brain cultures were inoculated overnight at 37°C with stock chimeric viruses at 20 ng of p24^{gag} per ml (measured by the Coulter p24 Antigen Assay Kit [SRA Technologies, Rockville, Md.]). After viral adsorption, the brain cultures were washed and refed every 3 to 4 days while the supernatants were collected to measure p24^{gag} release into the medium. Both NFN-SX and NFN-SM chimeric viruses produced cytopathic effects in microglial cells identical to those induced by uncloned JRFl, although with a somewhat slower time course. NFN-SM induced more pronounced cytopathic effects and a more rapid rise in p24 antigen than did NFN-SX, but both chimeric viruses caused production of as much as 30 ng of p24 antigen per ml in the supernatant (Fig. 1). In contrast, neither NFN-MX nor NF-Xho produced any cytopathic effects or any significant amount of p24 antigen in the culture supernatant compared with mock-infected cultures (Fig. 1). All four chimeric viruses, however, grew well in phytohemagglutinin-stimulated PBLs (grown as described in reference 16), causing release of comparable amounts of p24 antigen, usually reaching a peak at 14 days (Table 1). Immunofluorescence analysis confirmed the presence of p17^{gag} antigen in microglial cells of cultures infected with NFN-SM (Fig. 2) or NFN-SX, while no viral antigen was detected in such cells after inoculation with NF-Xho and NFN-MX (Fig. 2). In addition, the chimeric viruses produced by these human microglial cells were shown to be infectious for fresh PBLs from a seronegative donor (assayed as described in reference 4), as well as for fresh human brain cultures (Table 1). In contrast, supernatants of NF-Xho- or NFN-MX-inoculated brain cultures did not contain virus infectious for these cells (Table 1). Thus, a 158-aa

TABLE 1. Infectivity of chimeric viruses

Strain	p24^{gag} produced by PBL[a] at p.i. day:			Peak value in PBL[b]	p24^{gag} produced by microglia[a] at p.i. day:			Peak value in PBL
	6	15	21		7	14	21	
NFN-SX	0.6 ± 0.2	7.0 ± 2.3	3.0 ± 1.0	>30	0.16	2.1	5.4	>30
NF-Xho	<0.05	<0.05	<0.05	20.5	<0.05	<0.05	<0.05	<0.05
NFN-SM	10.0 ± 4.4	23.0 ± 5.9	10.1 ± 3.5	>30	0.09	1.9	6.3	>30
NFN-MX	<0.05	<0.05	<0.05	>30	<0.05	<0.05	<0.05	<0.05

[a] PBL supernatants were normalized with respect to p24^{gag} content; 20 ng was used per culture for inoculation of primary brain cultures, and 30 ng was used per culture of PBLs. PBL or human brain tissue cultures were inoculated at 1:2 with 16-day-postinoculation (p.i.) microglial supernatants whose p24 values were as follows (in nanograms per milliliter): NFN-SX, 4.1; NF-Xho, <0.01; NFN-SM, 7.1; NFN-MX, <0.01. The values shown are the means ± standard errors of three to six experiments per strain, with three to five duplicate cultures in each experiment. The magnitude of the standard errors, up to 44%, reflects donor-to-donor variation, as well as differences in the number of microglia cultured from a given lobectomy.

[b] Representative peak p24^{gag} values for infected PBLs are shown; they are not necessarily values for PBLs from the same donor. For infection of lymphocytes, the phytohemagglutinin-stimulated PBLs were stimulated with 2 μg of PHA-L (Dakopatts, Glostrup, Denmark) per ml, washed, and suspended in RPMI–15% fetal bovine serum–20 U of interleukin-2 (Boehringer Mannheim, Indianapolis, Ind.) per ml (16). These cells were then inoculated in 24-well plates at 5 × 10^5 per well (4). In each well, 30 ng of p24 was used for the overnight inoculations in a total volume of 1 ml per well. After incubation with the virus, 50% of the medium was replaced with 100% fresh medium and the cells were refed twice weekly by removing 50% of the supernatant, which was used for p24^{gag} analysis, and replacing it with fresh medium.

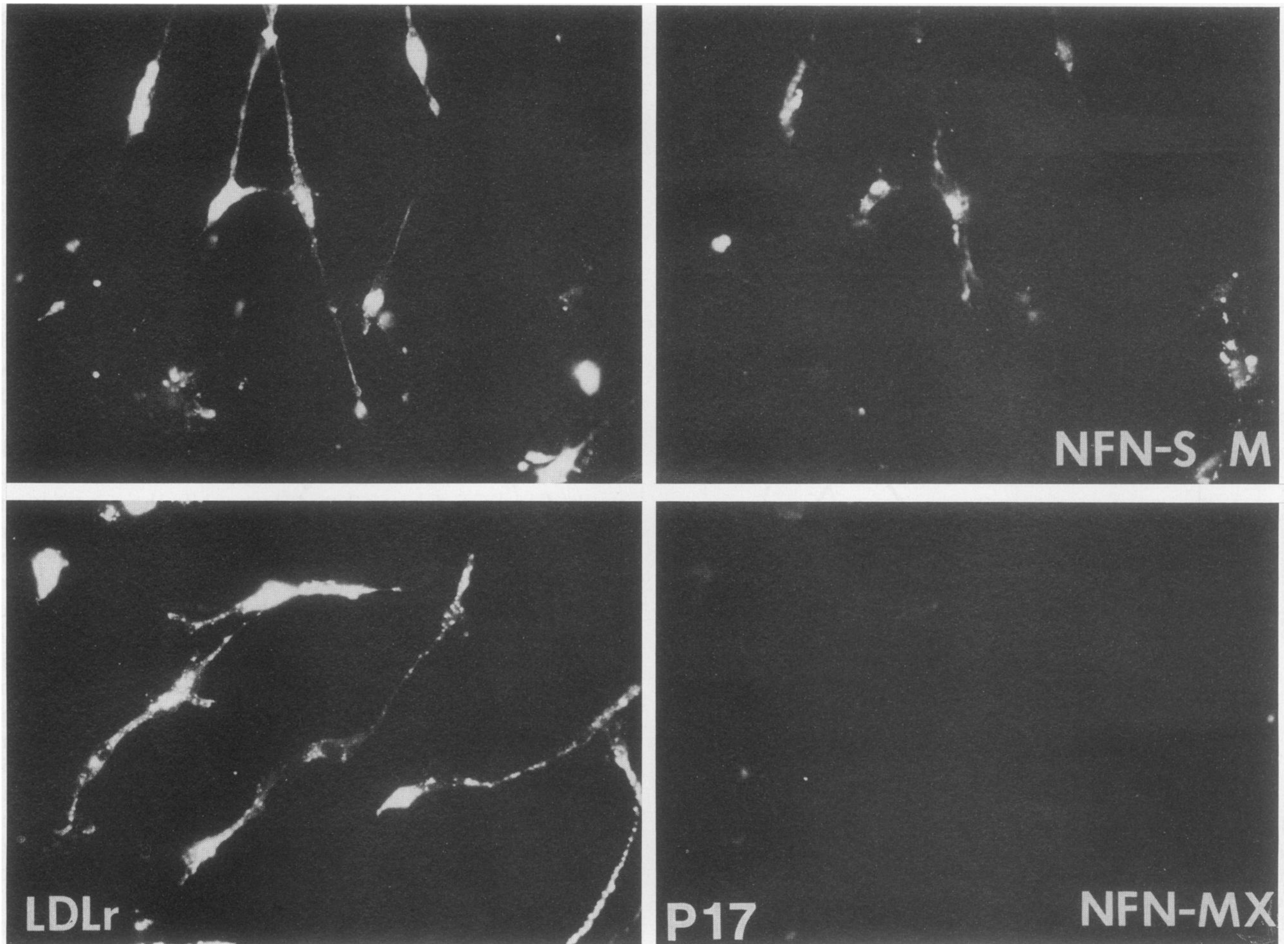
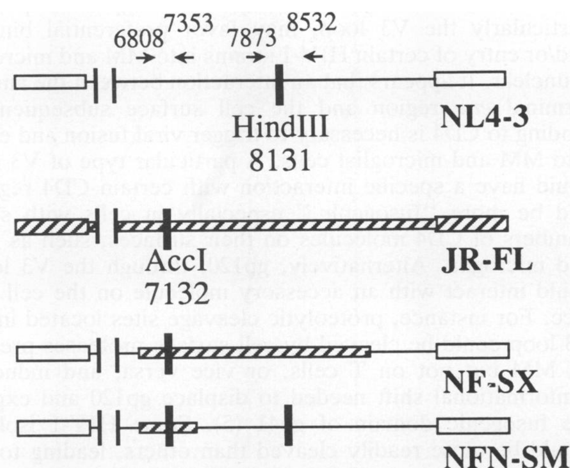


FIG. 2. Double immunofluorescence staining for the low-density lipoprotein receptor (LDLr) (left), which labels microglia and p17[gag] (right) in primary cultures (35) inoculated 15 days earlier with chimeric viruses NFN-SX and NFN-SM. Specific p17 staining was associated mostly with microglial cells and detected only with NFN-SX and NFN-SM chimeras, correlating well with the p24 antigen release shown in Fig. 1. Magnification, ×210.

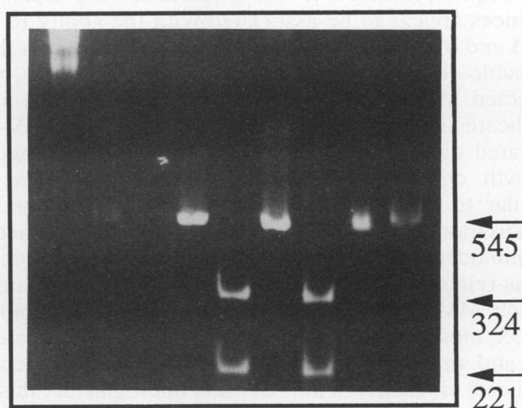
region of JRFl *env* (including the V3 loop) is sufficient to confer on a T-cell-tropic strain of HIV-1 the ability to enter and/or replicate efficiently in cultured microglial cells derived from adult human brain tissue.

Proviral DNA analysis of the chimeric viruses. To verify that the chimeric viruses that replicated in human microglia were identical to those inoculated, we prepared proviral DNA of brain cultures inoculated with NFN-SM and NFN-MX viruses pretreated with DNase as previously described (34, 37). (Control cultures were inoculated with virus that had been heat inactivated at 65°C for 1 h.) DNA from brain cells grown in two 60-mm-diameter plastic dishes per strain—each containing 5×10^5 microglia, as well as other proliferating cell types—was harvested at 21 days postinoculation for restriction analysis, yielding approximately 10 to 60 μg of DNA. We then amplified by polymerase chain reaction (PCR) (28) two different regions that contained restriction sites (*Acc*I and *Hin*dIII) which were not conserved between the two chimeric viruses (Fig. 3). To bracket the regions containing these sites, we used primers located in the gp120- and gp41-coding regions. The primer sequences used were as follows: gp120 sense, CTC AGT CAT TAC ACA GGC CTG [6808]; gp120 antisense, CCT CCA CAA TTA AAA CTG TGC [7353]; gp41 sense, CAA TTT GCT GAG GGC TAT TGA GGC GC [7873]; gp41 antisense, TCT CTC AAG CGG TGG TAG CTG AAG AGG [8532]. PCR amplification was run for 35 cycles of 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min in PCR buffer containing 1.5 mM $MgCl_2$ in a Perkin Elmer PCR machine. PCR products were then digested with 20 to 30 U of each restriction enzyme (New England BioLabs, Beverly, Mass.) at 37°C overnight. Fragments resulting from the digestion were visualized by ethidium bromide staining of 1.5% agarose gels (Fig. 3). This analysis confirmed the composition of the proviral DNA of each chimera in the infected cells, indicating that the viruses replicating in our cultures were indeed NFN-SX and NFN-SM (Fig. 3).

To determine whether infection by the chimeras with T-cell-tropic *env* sequences is blocked at a stage prior to formation of proviral DNA, we also purified DNA of primary brain cultures inoculated 7 days earlier with each recombinant by using a set of primers (M661 and M667 [37]) corresponding to the 3′ region of the long terminal repeat and the early region of *gag*—identical in all four chimeras—in a sensitive PCR assay using end labeling of M661 (24, 37). As predicted, a high number of proviral DNA copies was



Digestion with AccI



Digestion with HindIII

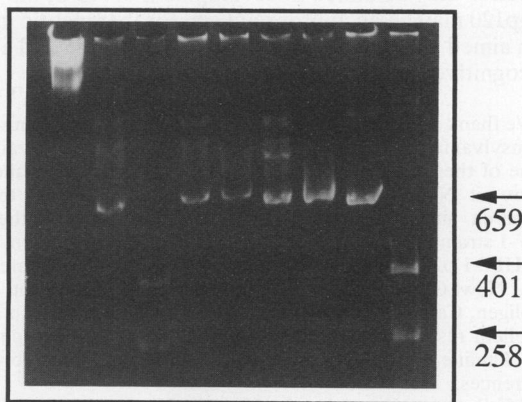


FIG. 3. Restriction enzyme analysis of DNA from brain tissue cultures infected with NFN-SX and NFN-SM chimeras. PCR-amplified fragments of proviral DNAs of NFN-SX and NFN-SM were digested with restriction enzymes whose sites were not conserved between the NL4-3 and JRFl genomes. The primers used are shown above the NL4-3 genome, and the base pair numbers correspond to their locations in NL4-3 (22). The amplified fragment (6808 to 7353) in the coding region of gp 120 contains an *Acc*I site in JRFl at 7128, but this site is absent in NL4-3. Likewise, the amplified fragment (7873 to 8532) in the coding region of gp41 contains a *Hin*dIII site in NL4-3 at 8131 which is not present in JRFl. As a result, both chimeras were digested by *Acc*I while only NFN-MX was digested by *Hin*dIII.

detected in human brain cultures infected with NFN-SM and NFN-SX while no significant number of proviral copies—compared with the heat-inactivated virus control—was detected in cells inoculated with NF-Xho or NFN-MX (data not shown). Thus, the *env* gene of T-cell-tropic strain NL4-3 lacks determinants necessary for the early stage of the virus cycle in human brain microglia.

Entry of microglia-tropic chimeras is CD4 mediated, but the V3 loop of gp120 determines tropism. To test whether the microglia-tropic chimeric viruses use the CD4 receptor for viral entry, human brain cultures were incubated at 4°C with the OKT4A antibody (at 20 μg/ml; Ortho Diagnostics, Raritan, N.J.), which recognizes the gp120-binding site on the CD4 molecule (29). This was followed by virus adsorption in the cold (15). This antibody pretreatment completely blocked infection with NFN-SX virus, as assayed by p24 antigen release into the supernatant over a 3-week period; it also considerably slowed down infection with NFN-SM, resulting in $p24^{gag}$ levels eight times lower than those of the untreated cultures (data not shown). Thus, both microglia-tropic strains seemed to use CD4 to enter microglial cells, even though the putative CD4-binding site on gp120 is derived from parental strains with different microglial cell tropisms. However, these strains differ by only one residue in this region (residues 410 to 454 of JRFl [23]). In NF-Xho and NFN-SM, the CD4-binding sites in the carboxy-terminal portion of gp120 are identical (Fig. 1), yet only NFN-SM replicates in microglia. Thus, it appears that in NF-Xho and NFN-SM the CD4-binding site on gp120 cannot be a determinant of tropism.

We next examined whether specific JRFl sequences within the V3 loop are essential in determining tropism. The V3 loop is an immunodominant region and a binding site for neutralizing antibodies that do not block binding of gp120 to CD4 and is involved in fusion (9, 27, 33). We preincubated the chimeric virus NFN-SM at 37°C for 2 h with human monoclonal antibody N-710.9B N (31) at 5 and 25 μg/ml before adsorbing virus onto the human brain cells. This antibody recognizes the GPGRAFYTT sequence in a 13-aa epitope of the V3 region of the MN strain which differs by only 1 aa from the JRFl V3 sequence (31). No evidence of $p17^{gag}$ expression was found by immunofluorescence after 12 days in cultures inoculated with the antibody-treated virus, while several foci of infected microglial cells were readily detected in cultures infected with untreated virus (data not shown). Furthermore, brain cultures inoculated with the V3 antibody-treated chimeric virus released no significant amounts of p24 antigen into the supernatant, in contrast to untreated cultures (Fig. 4). The blocking effect of this V3 antibody was abrogated by the homologous V3 peptide (MN strain, RP142) but not by an unrelated V3

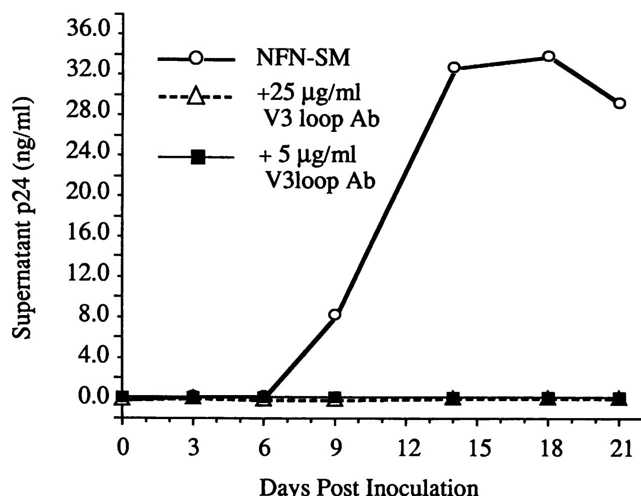


FIG. 4. Infection of human brain tissue cultures with NFN-SM is blocked by preincubation of the virus with an antibody (Ab; 5 or 25 μg/ml) that recognizes the V3 loop within the *env*-encoded protein. The cultures were then incubated with both virus and antibodies overnight at 37°C, washed three times, and refed every 3 to 4 days. Infection was assayed by release of p24 antigen into the supernatant of the cultures over time. Each point represents the values for the pooled supernatant of three cultures.

peptide (IIIB strain, RP135) (13) (data not shown). Entry of the NFN-SM chimera into PBLs was also inhibited by this V3 antibody, while LAV replication was unaffected (as described in reference 31), indicating that the inhibition was virus specific. Thus, the V3 loop appears to be crucial in cell surface interactions, leading to efficient viral entry into human brain microglial cells by a chimeric virus expressing 158 aa of JRFl gp120.

The present study demonstrated that a discrete region of the *env* gene of an HIV-1-neurotropic strain (JRFl) outside the CD4-binding site can confer on a T-cell-tropic virus the ability to enter and replicate in human brain microglial cells. Similar discrete regions in the HIV-1 gp120 glycoprotein have been shown to determine MM tropism; they consist of a gp120 stretch of 159 aa in the SF162 strain (2), 94 residues in the ADA strain (36), and only 20 aa within the V3 loop of the BaL strain (12). Interestingly, the V3 loops of both the JRFl and ADA strains have the exact same sequence as the V3 consensus sequence (18) while the BaL strain differs only by 1 aa and the SF162 strain differs by three residues from this consensus. Furthermore, the deduced amino acid sequence of the V3 loop of a HIV-1 clone derived directly from the brain of a patient with ADC contains only 2 aa differences from the consensus (21). Compared with the T-cell strain sequences, the maximum number of substitutions in the V3 loop (10 aa) is seen between the BaL and HTLV-IIIB strains (12). Interestingly, all of these MM-tropic strains have a tyrosine at the third residue after the GPGR tip of the V3 loop while none of the three T-cell-tropic strains has a tyrosine in this position (36). The JRFl V3 loop—within the *env* chimeras used in the present study—differs from the NL 4-3 V3 loop by 7 aa, and 6 of these are located on the carboxyl-terminal side of the V3 loop. Thus, these changes may be responsible for efficient entry of NFN-SX and NFN-SM chimeras into MM and microglial cells although the chimeric viruses show another 16 aa differences between JRFl and NL4-3 outside the V3 loop.

The mechanism by which a particular gp120 sequence, particularly the V3 loop, may favor preferential binding and/or entry of certain HIV-1 strains into MM and microglia is unclear. It appears that an interaction between the amino-terminal *env* region and the cell surface subsequent to binding to CD4 is necessary to trigger viral fusion and entry into MM and microglial cells. A particular type of V3 loop could have a specific interaction with certain CD4 regions and be more "fusogenic," especially in cells with small numbers of CD4 molecules on their surfaces, such as MM and microglia. Alternatively, gp120, through the V3 loop, could interact with an accessory molecule on the cell surface. For instance, proteolytic cleavage sites located in the V3 loop could be cleaved by cell surface proteases present on MM but not on T cells, or vice versa, and induce a conformational shift needed to displace gp120 and expose the fusogenic domain of gp41 (5). Some HIV-1 isolates would be more readily cleaved than others, leading to the observed differences in infectivity for different cell types.

In patients with AIDS, wild-type PBL isolates often show V3 sequences close to the consensus (18) and such sequences appear to be associated with the ability to grow in MM and brain microglia, as discussed above. It is therefore possible that such viruses are carried into the brain by infected MM and/or T cells early in the disease and then replicate in the resident microglial cells. An HIV-1 clone isolated directly from brain tissue without passage shows growth characteristics and a V3 loop sequence closely similar to those of the MM-tropic HIV-1 strains (21). In rhesus monkeys inoculated with a molecularly cloned simian immunodeficiency virus strain, simian immunodeficiency virus-related encephalitis occurred only in those animals in which MM-tropic variants arise in vivo (6). Similarly, in AIDS, most brain isolates have the ability to replicate in MM (3) and only MM-tropic strains of HIV-1 can enter and replicate efficiently in human brain microglia (35; this study). HIV-1 probably enters nervous tissue in T cells and/or MM and then spreads and replicates through the microglial cell network in the brain parenchyma. We have shown here that JRFl tropism for brain-derived microglial cells is controlled by *env* determinants similar to those that determine MM tropism and that the V3 loop is a critical factor in this tropism. Since microglial cells are the major cell type in which HIV-1 replicates in the adult brain and since their infection may be linked to development of ADC, the V3 loop of gp120 may be an appropriate site for therapeutic intervention aimed at stopping virus spread in the brain and a decline in cognitive function.

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