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Genetic and Functional Analysis of Full-Length Human Immunodeficiency Virus Type 1 *env* Genes Derived from Brain and Blood of Patients with AIDS

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The genetic evolution of human immunodeficiency virus type 1 (HIV-1) in the brain is distinct from that in lymphoid tissues, indicating tissue-specific compartmentalization of the virus. Few primary HIV-1 envelope glycoproteins (Envs) from uncultured brain tissues have been biologically well characterized. In this study, we analyzed 37 full-length *env* genes from uncultured brain biopsy and blood samples from four patients with AIDS. Phylogenetic analysis of intrapatient sequence sets showed distinct clustering of brain relative to blood *env* sequences. However, no brain-specific signature sequence was identified. Furthermore, there was no significant difference in the number or positions of N-linked glycosylation sites between brain and blood *env* sequences. The patterns of coreceptor usage were heterogeneous, with no clear distinction between brain and blood *env* clones. Nine Envs used CCR5 as a coreceptor, one used CXCR4, and two used both CCR5 and CXCR4 in cell-to-cell fusion assays. Eight Envs could also use CCR3, CCR8, GPR15, STRL33, Apj, and/or GPR1, but these coreceptors did not play a major role in virus entry into microglia. Recognition of epitopes by the 2F5, T30, AG10H9, F105, 17b, and C11 monoclonal antibodies varied among *env* clones, reflecting genetic and conformational heterogeneity. Envs from two patients contained 28 to 32 N-glycosylation sites in gp120, compared to around 25 in lab strains and well-characterized primary isolates. These results suggest that HIV-1 Envs in brain cannot be distinguished from those in blood on the basis of coreceptor usage or the number or positions of N-glycosylation sites, indicating that other properties underlie neurotropism. The study also demonstrates characteristics of primary HIV-1 Envs from uncultured tissues and implies that Env variants that are glycosylated more extensively than lab strains and well-characterized primary isolates should be considered during development of vaccines and neutralizing antibodies.

Human immunodeficiency virus type 1 (HIV-1) infects macrophages and microglia in the central nervous system (CNS) and frequently causes dementia and other neurological disorders. HIV-1 enters the CNS in the early stages of infection by trafficking across the blood-brain barrier within infected monocytes and possibly lymphocytes (14). However, CNS infection is typically latent, and HIV-1-associated dementia usually occurs only after progression to AIDS (reviewed in references 11 and 14). The genetic evolution of HIV-1 within the brain is distinct from that in lymphoid tissues and other organs (5, 20, 21, 44). The genetic compartmentalization of viral variants in the CNS suggests that adaptive changes occur in response to unique constraints of the CNS microenvironment, such as different target cell populations and immune selection pressures. However, the biological characteristics of primary envelope glycoproteins (Envs) in brain are not well defined, and YU-2 (24) is the only full-length HIV-1 Env from uncultured brain tissue that has been biologically well characterized.

The tropism of HIV-1 is determined by the interaction of the viral Envs with CD4 and a coreceptor (reviewed in reference 3). Macrophage-tropic HIV-1 viruses primarily use CCR5 (R5) as a coreceptor, whereas T-cell line-tropic viruses use CXCR4 (X4). Dual-tropic viruses (R5X4) use both coreceptors. A subset of viruses can also use alternative coreceptors, such as CCR2b, CCR8, Apj, STRL33 (BONZO/CXCR6), GPR1, GPR15 (BOB), CX3CR1 (V28), Chem R23, and RDC-1, for virus entry in transfected cells. In some patients, HIV-1 disease progression is associated with broadening of virus tropism by expansion of coreceptor usage and emergence of X4 or R5X4 variants (3). However, previous studies suggest that usage of coreceptors other than CCR5 and CXCR4 by primary viruses is rare (49) and infection of primary cells occurs, with few exceptions (23), exclusively via CCR5 or CXCR4 (49). CCR5 is the major coreceptor for HIV-1 infection of macrophages and microglia (1, 15, 18, 38) and the principal coreceptor used by HIV-1 viruses isolated from brain (1, 15, 16, 18, 38). However, macrophages and microglia can also support efficient replication by a subset of X4 viruses (16, 30), and macrophage tropism predicts HIV-1 neurotropism independent of coreceptor specificity (16). Thus, neurotropism is governed by factors other than coreceptor usage.

Most previous studies have characterized biological properties of full-length HIV Envs cloned from passaged virus iso-

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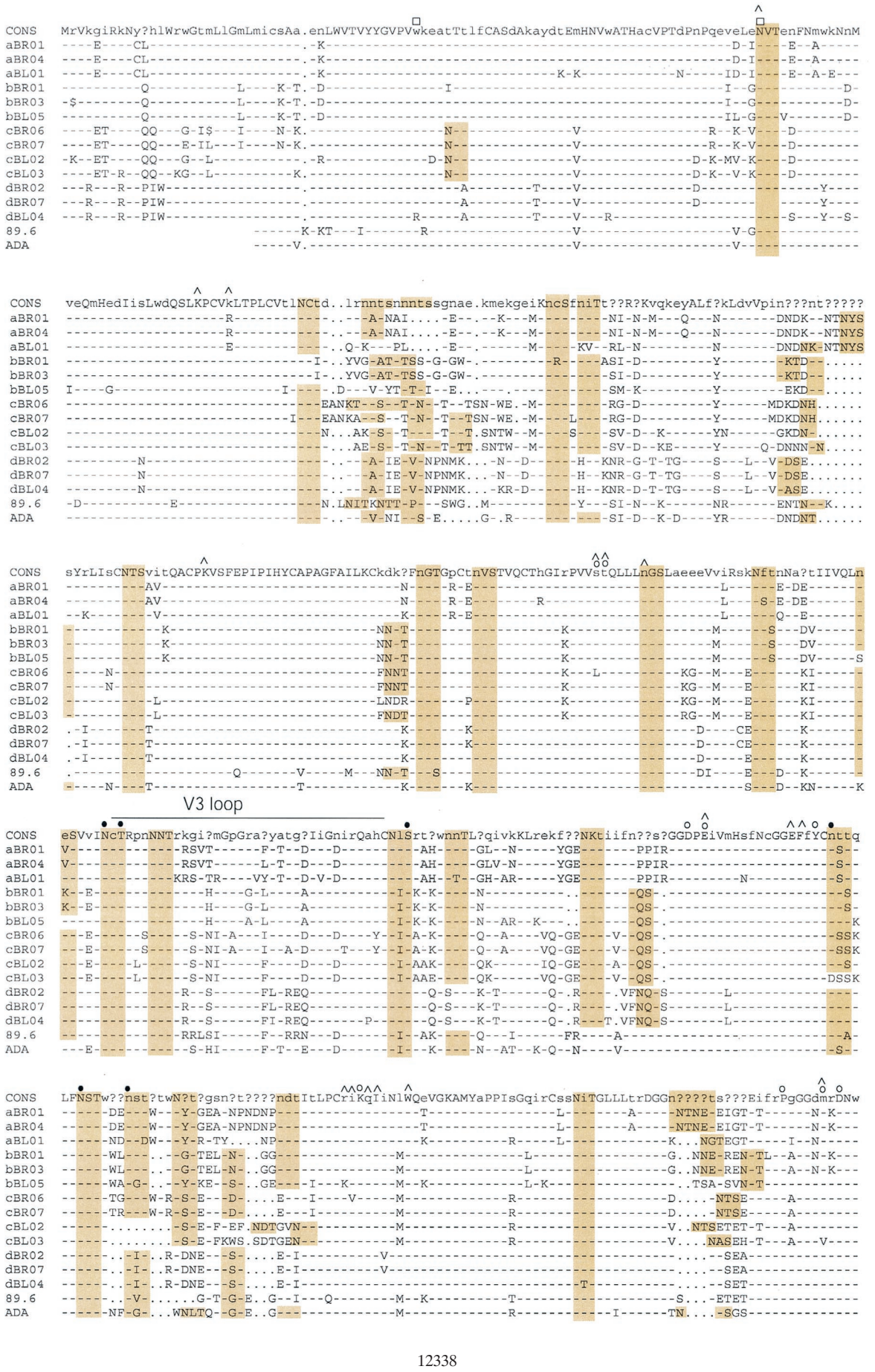
lates rather than from uncultured tissues (13, 19, 39), but there are a few exceptions (24, 32). To gain a better understanding of genetic and biological characteristics of HIV-1 Envs in uncultured brain tissue, full-length HIV-1 *env* genes were cloned directly from uncultured brain biopsy samples from patients with late-stage AIDS and their sequences and functional characteristics were compared with those of *env* genes cloned from peripheral blood samples.

Sequencing and phylogenetic analysis of HIV-1 *env* genes from brain and blood. Thirty-seven full-length HIV-1 *env* genes were cloned directly from blood and brain biopsy samples from four patients with late-stage AIDS and CD4 counts ranging from 1 to 39 cells/mm^3. The study subjects were selected from AIDS patients scheduled to undergo a diagnostic stereotactic brain biopsy. Written informed consent was obtained from the patient or legal guardian. The Institutional Review Board of Northwestern University approved the protocol. No patient had neurological symptoms prior to the presenting acute neurological symptoms that prompted a brain biopsy. Brain biopsy results were diagnostic of primary CNS lymphoma. Peripheral blood collected during the operation and brain biopsy cores contiguous to, but not overlapping, the lymphoma lesion were stored at −135°C. Full-length HIV-1 *env* genes were amplified from genomic DNA at end point dilution by nested PCR. Peripheral blood mononuclear cells (PBMC) were obtained from whole blood by Ficoll-Hypaque density gradient centrifugation. Nested PCR was performed in a Perkin-Elmer 9600 thermocycler (1 cycle at 95°C for 1 min, 40 cycles at 95°C for 15 s and at 60°C for 10 min, and a final extension at 72°C for 7 min) using RTth XL polymerase (Perkin-Elmer). The outer primers were *env*1A and *env*1M (13). The inner primers were *env*1B (13) and *env*1L (5′-TTTTGAC CACTTGCCACCCAT-3′). PCR products were gel-purified, cloned into pAMP1 (GIBCO-BRL), and sequenced in both directions.

Figure 1 shows an alignment of the predicted amino acid sequences for 13 *env* clones in which the coding potential was maintained. Potential inactivating mutations (i.e., premature stop codons and/or frameshifts) were observed in most of the other *env* clones. Phylogenetic comparisons were done based on various breakdowns of the PCR product sequence data sets as previously described (21, 46). Phylogenetic analysis of intrapatient sequence sets showed that brain-derived *env* sequences were tightly clustered relative to blood-derived *env* sequences, indicating tissue-specific compartmentalization of the virus (Fig. 2). A signature pattern analysis was done to search for possible amino acid positions that would provide a conserved pattern within the brain *env* sequences relative to the blood *env* sequences. The first approach calculated (with VESPA software) the frequency of an amino acid at a specific position and then determined whether there was a distinct pattern for one set of sequences (i.e., blood or brain). The second approach used Shannon entropy (with ENTROPY software) to calculate the consistency of an amino acid at one specific position. To assess the statistical significance of the most distinctive motifs identified, a Monte Carlo-like randomization of viral sequences was used to test the statistical significance of the signature pattern (21). MotifScan was then run on each of x randomized sets to determine the background level of distinctive motifs, i.e., what is typically observed by chance alone. No convincing signature pattern was associated uniquely with brain or blood sequences. We also compared the number of potential N-linked glycosylation sites between brain and blood *env* sequences by the Wilcoxon rank-sum test and found no significant difference between the two sets of sequences. Furthermore, there was no significant difference in the positions of N-linked glycans. Thus, no brain-specific signature sequence was identified.

Env expression and processing. To functionally characterize Envs with uninterrupted gp160 coding regions, 13 *env* genes were subcloned into the pSVIIIenv expression plasmid (13) by replacement of the 2.1-kb *Kpn*I to *Bam*HI HXB2 *env* fragment. These Env plasmids contain the entire gp160 coding region of blood- and brain-derived *env* genes except for 36 amino acids at the N terminus and 105 amino acids at the C terminus, which are derived from HXB2. pAMP1 Env plasmids were used as templates to amplify the *env* coding sequences by PCR with 5′ *Kpn*I and 3′ *Bam*HI restriction sites (nucleotides 6,348 to 8,478 in HXB2) by using the 5′ primer (5′-GTCTATTAT GGGGTACCTGTGTGG-3′) and the 3′ primer (5′-GCTA AGGATCCGTTCACTAATCGT-3′) (18). An internal *Kpn*I site in the aBR-01 *env* was eliminated without changing any amino acids by using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The pSVIIIenv plasmids were sequenced to confirm that no errors were introduced during the PCR amplification.

To analyze Env expression and processing, 293T cells were transfected with 10 μg of the Env plasmids and cell lysates were analyzed by Western blotting as previously described (17). The 89.6 Env (7) was used as a positive control. 293T cells transfected with empty vector plasmid were used as negative controls to detect nonspecific background bands reacting with each antibody. The results are summarized in Table 1. Western blotting with anti-gp120 (American Biotechnologies, Inc.) showed that 3 *env* clones expressed distinct gp160 and gp120 proteins, whereas 10 expressed one band at approximately 140 to 170 kDa, representing either unprocessed gp160 or gp160 and gp120 proteins that migrated at similar positions (data not shown). Western blotting was then performed with antibodies directed against gp41. The monoclonal antibody 2F5 (National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) (4, 34, 35), which is directed against the epitope NEQELLELDKWASLWN in the gp41 ectodomain (4, 31, 34, 35), detected bands corresponding to gp160 and gp41 for *env* clones from patients A, C, and D. The level of gp41 expression detected by 2F5 was highly variable, with seven *env* clones expressing strong gp41 bands and three expressing weak gp41 bands. The strong bands detected at approximately 35 kDa for the cBL-02 and cBL-03 Envs represent truncated gp41 proteins (Fig. 1). The T30 antibody (provided by C. Broder), which is directed against a glycosylation-dependent epitope in the gp41 ectodomain (9), detected all of the gp160 proteins (data not shown) and 10 of the gp41 proteins (Table 1). The AG10H9 antibody (BabCO), which is directed against amino acids 717 to 751 of gp41 (FQTLIPNPTEADRP GGIEEGGGEQGRTRSIRLVNG), detected gp160 and/or gp41 proteins expressed by several *env* clones from patients A, B, and D but not patient C. Thus, Env proteins from patients A, B, C, and D exhibited significant variability in the processing efficiency of gp160 and in several gp41 epitopes.


V3 loop

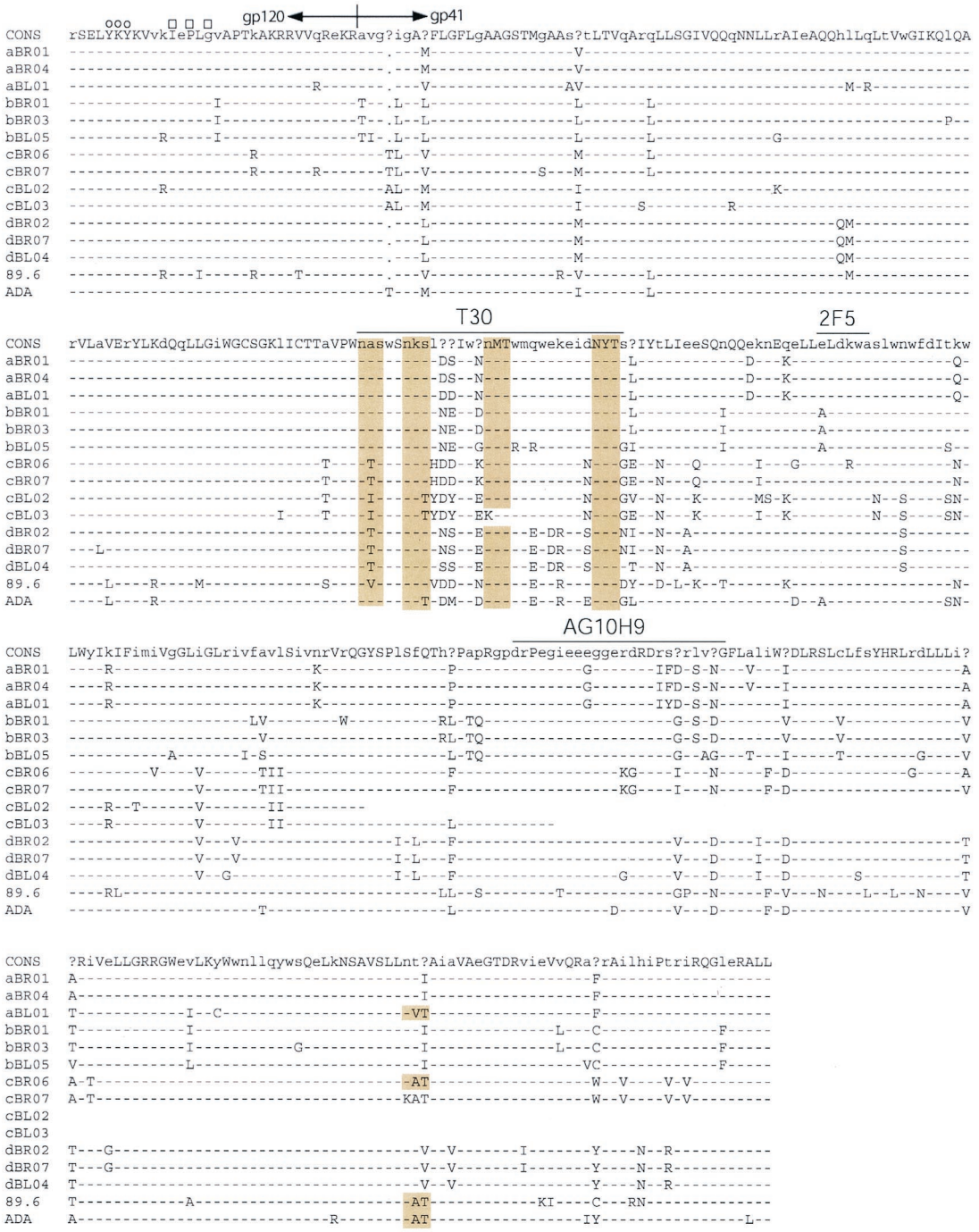


FIG. 1. Amino acid alignment of primary HIV-1 Env clones from the blood (BL) and brains (BR) of four patients with AIDS (patients A through D) compared to that of the clade B consensus and to the 89.6 and ADA Env clones. Potential N-linked glycosylation sites are marked by shaded boxes. Epitopes recognized by monoclonal antibodies used for Western blotting, immunoprecipitation, and neutralization studies are underlined or indicated by the following symbols: open circle, F105; caret, 17b; open square, C11; filled circle, 2G12. The F105, 17b, C11, and 2G12 epitopes are discontinuous. Mutagenesis studies have shown that these monoclonal antibodies are sensitive to changes in gp160 at the following positions: (i) F105 is sensitive to changes at 256S, 257T, 368D, 370E, 384Y, 421K, 470P, 475M, 477D, 482Y, 483Y, and 484Y; (ii) 17b is sensitive to changes at 88N, 117K, 121K, 207K, 256S, 257T, 262N, V3 loop, 370E, 381E, 382F, 419R, 4201, 421K, 422Q, 432I, 427W, 435Y, 438P, and 475M; (iii) C11 is sensitive to changes at 45W, 88N, 491I, 493P, and 495G; and (iv) 2G12 is sensitive to changes at 295N, 297T, 334S, 386N, 392N, and 397N (reviewed in reference 47).

We performed immunoprecipitation assays using a panel of conformation-sensitive gp120 monoclonal antibodies (2G12, F105, 17b, and C11). 293T cells transfected with the different Envs were lysed under mild detergent conditions (0.5% NP-40) previously shown to preserve the folding of monomeric Env proteins (42). Env proteins were immunoprecipitated with 4 μg of 2G12, F105, 17b, or C11 antibodies and protein A-Sepharose. The 2G12, F105, and 17b monoclonal antibodies

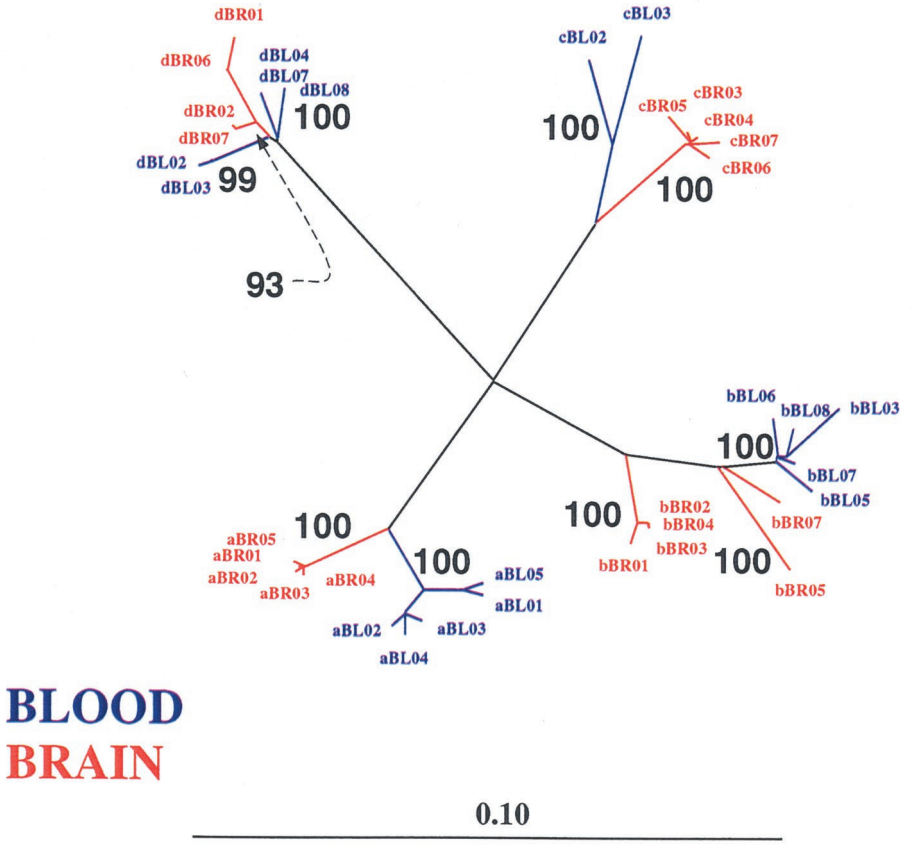


FIG. 2. Phylogenetic analysis of full-length HIV-1 envelope gene sequences in blood and brain. The image is a PHYLIP neighbor-joining tree based on the F84 model used for PHYLIP maximum-likelihood trees. The color-coded regions represent the full-length gp160 *env* sequences cloned directly from blood and brain tissue from four patients with AIDS (patients A through D). The numbers associated with each branch represent their bootstrap values. Bootstrap resampling was done by using 100 replicates.

(4, 33, 41, 43, 47) were obtained from the NIH AIDS Research and Reference Reagent Program, and the monoclonal antibody C11 (28) was provided by R. Wyatt. 2G12 is directed against a unique epitope in the C3V4 region (43, 47). F105 is directed against a discontinuous epitope overlapping the CD4-binding surface on gp120 (33, 47). 17b recognizes an epitope overlapping the coreceptor binding site (22, 36). C11 is directed against an epitope that includes the C1 and C5 regions of gp120 (28). The immunoprecipitates were washed, boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, separated on sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis gels, and analyzed by Western blotting using rabbit anti-gp120 serum. The results are summarized in Table 1. All Envs were efficiently precipitated by the 2G12 antibody. In contrast, the F105, 17b, and C11 antibodies immunoprecipitated only a subset of Envs. Together, 7 of 13 Envs were efficiently immunoprecipitated by all four gp120 antibodies, suggesting correct folding of the CD4- and coreceptor-binding regions, the C1 and C5 regions, and the C3V4-spanning 2G12 epitope.

Coreceptor usage. Env function and coreceptor usage was examined in syncytium and infection assays. The 89.6 and ADA Envs were used as positive controls. Eleven Envs mediated cell-to-cell fusion with at least one coreceptor, with two of these weakly positive (Table 2). All fusion-competent Envs

TABLE 1. Results of Western blotting and immunoprecipitation of Envs with gp120 and gp41 antibodies[a]

Envelope	gp41 Western blotting			gp120 immunoprecipitation				gp160 processing
	2F5	T30	AG10H9	2G12	F105	17b	C11	
aBR-01	+	+	+	+	+	+	+	++
aBR-04	+/−	−	+/−	+	−	−	−	+/−
aBL-01	+/−	−	+	+	−	+	+	+
bBR-01	−	+	+/−	+	+/−	+	−	+/−
bBR-03	−	−	−	+	+	+	+	−
bBL-05	−	−	−	+	+	+	+	−
cBR-06	−	+	−	+	−	−	−	+
cBR-07	+	+	−	+	+	+	+	+
cBL-02	+	+	−	+	+	+	+	+
cBL-03	+	+/−	−	+	+	−	+	+
dBR-02	+	+/−	+/−	+	+	+	+	++
dBR-07	+	+	−	+	+	+	+	++
dBL-04	+/−	+	−	+	−	−	−	+/−
89.6	+	+	+	+	+	+	+	++

[a] 293T cells were transfected with Env plasmids, and cell lysates were analyzed by Western blotting or immunoprecipitated with the indicated monoclonal antibodies. Epitopes recognized by these antibodies are described in the text and/or indicated in Fig. 1. The levels of gp41 and gp120 detected by Western blotting and immunoprecipitation, respectively, are indicated as follows: −, +/−, and + represent levels that were, respectively, absent, lower than, or similar to levels detected for the 89.6 Env. The efficiency of gp160 processing was determined by Western blotting with rabbit anti-gp120 and three gp41 monoclonal antibodies (2F5, T30, and AG10H9) and is indicated as follows: −, +/−, +, and ++ represent, respectively, an undetectable level, a 3- to 4-fold reduction, a 1.5- to 2-fold reduction, and no reduction compared to the efficiency of g160 processing detected for the 89.6 Env.

TABLE 2. Results for coreceptors used in syncytium and infection assays

Envelope	Syncytium assays[a]						Infection assays[b]								
	CXCR4	CCR5	CCR3	Apj	STRL33	GPR1	CXCR4	CCR5	CCR3	Apj	STRL33	GPR1	CCR2b	GPR15	CCR8
Original clones															
aBR-01	+	+++	+	+/−	+	+/−	+	+++	+/++	+/−	−	+	+/−	+/−	+/−
aBR-04	−	+	+	−	−	−	−	+/−	−	−	−	−	−	−	−
aBL-01	+++	+	+	+	+	+	+++	++	+	+/−	+/−	+	+/−	+/−	−
bBR-01	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
bBR-03	−	+/−	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
bBL-05	−	+/−	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
cBR-06	−	+/++	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
cBR-07	−	+/++	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
cBL-02	−	++	+	−	−	−	−	−	−	−	−	−	−	−	−
cBL-03	−	+	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
dBR-02	+++	+++	++	+/++	+/−	+	+++	+++	++	+	+	+	−	+/++	+
dBR-07	+++	+++	++	+/++	+/−	+	+++	+++	++	+	+	+	−	+/++	+
dBL-04	−	−	−	−	−	−	−	−	−	−	−	−	ND	ND	ND
89.6	++	++	+/++	+	+	−	+++	++	+	+	−	−	+/−	−	−
ADA	−	+++	+/++	−	+	−	−	++/+++	++	+/−	+/−	+	−	+/++	+
Mutant clones															
aBR-04-H	+	++	+	+/−	+	+/−	+	+++	+	−	+	+/−	ND	ND	ND
bBR-01-C	−	+/++	+	−	−	−	−	+	+	−	−	−	ND	ND	ND
bBR-01-L	−	+++	+/++	+	++	+	−	+++	+/++	+	+/++	−	ND	ND	ND

[a] Syncytium assays were performed by coculture of 293T cells cotransfected with Env and Tat plasmids with Cf2-Luc cells expressing CD4 and the indicated coreceptor as previously described (17). Fusion was determined by measurement of luciferase activity 8 h later and is indicated as follows: +/−, +, +/++, ++, and +++ represent results 1.5- to 2-fold, 2- to 5-fold, 5- to 10-fold, 10- to 20-fold, and >20-fold above background levels, respectively. Results less than 1.5-fold above the background level obtained with no Env were considered negative.

[b] Single-cycle infection assays were performed by infecting U87 cells expressing CD4 and the indicated coreceptor with luciferase reporter viruses (20,000 ^{3}H cpm reverse transcriptase units) for 60 h as previously described (17, 30). Infection was determined by measurement of luciferase activity and is indicated as follows: +/−, +, +/++, ++, and +++ represent results 10- to 20-fold, 20- to 50-fold, 50- to 100-fold, 100- to 500-fold, and 7,500-fold above background levels, respectively. Results less than 10-fold above the background level obtained with no Env were considered negative. ND, not determined.

used CCR5. Two brain-derived Envs (dBR-02 and dBR-07) used CCR5 and CXCR4 equally well, in addition to using CCR3, Apj, STRL33, GPR1, GPR15, and CCR8 at lower efficiency (Table 2 and data not shown). The ability of the Envs to mediate virus entry during a single round of infection was examined by using pseudotyped HIV-1 luciferase reporter viruses as previously described (17, 18). Five Envs (aBR-01, aBR-04, aBL-01, dBR-02, and dBR-07) were functional in infection assays (Table 2). The dBR-02 and dBR-07 Envs used both CCR5 and CXCR4 in addition to using alternative coreceptors at lower efficiency. The aBR-01 Env primarily used CCR5, with additional minor usage of CXCR4 and alternative coreceptors. The aBL-01 Env principally used CXCR4 but also showed moderate usage of CCR5 and minor usage of alternative coreceptors. The aBR-04 Env used CCR5 exclusively. None of these Envs was CD4 independent (data not shown). These results demonstrate heterogeneous patterns of coreceptor usage with no clear distinction between brain and blood *env* clones.

Viral replication and cytopathicity. To examine the ability of these Envs to mediate replication and cytopathic effects in PBMC and primary brain cultures, chimeric NL4-3 viruses containing the aBR-01, aBL-01, dBR-02, and dBR-07 Envs were constructed by subcloning the *Kpn*I to *Bam*HI *env* gene fragments into the NL4-3 plasmid. NL4-3 viruses that contain the ADA and 89.6 *env* genes (NL-ADA and NL-89.6, respectively) were used as positive controls. In PBMC, high levels of replication were observed for the aBL-01, dBR-02, and dBR-07 viruses, whereas the aBR-01 virus had slightly delayed replication kinetics (data not shown). In primary human brain cultures prepared as previously described (30), high levels of replication were observed for the aBR-01, dBR-02, and dBR-07 viruses, whereas the aBL-01 virus replicated to moderate levels (data not shown). Viruses with the dBR-02, dBR-07, and 89.6 Envs induced an approximately 200% increase in apoptosis in primary brain cultures measured as described previously (17) compared to that for mock-infected control cultures ($P < 0.05$), whereas viruses with the aBR-01, aBL-01, and ADA Envs induced minor increases in apoptosis that were not statistically significant (data not shown). Thus, two dual-tropic Envs derived from brain were cytopathic for CNS cells, a finding consistent with those of previous studies of dual-tropic viruses derived from blood and lymphoid tissues (17, 30).

Amino acid changes responsible for functional defects. We considered possible explanations for the inefficient Env processing and functional defects of several *env* clones by analyzing the amino acid sequences in detail (Table 3). The cBL-02 and cBL-03 Envs contain a premature stop codon in gp41, resulting in transmembrane proteins consisting of only 200 and 220 amino acids, respectively, raising the possibility that Env incorporation into virions might be defective (8, 10, 11). Twelve of 13 Envs had the 18 Cys residues in gp120 that are required to form the nine disulfide bridges. The bBR-01 Env lacks a Cys residue between the V1 and V2 loops (Fig. 1, position 158), suggesting that this amino acid change might cause these loops to misfold. The number of Cys residues in gp41 ranged from two to four, compared to five or seven Cys residues in the gp41 proteins of 89.6, ADA, and HXB2. Twenty-eight to 32 N-linked glycosylation sites were present in gp120 proteins from patients B and C, compared to 23 to 25 for gp120 proteins from patients A and D, 24 for gp120 from

TABLE 3. Analysis of HIV-1 envelope amino acid sequences

HIV-1 Env	No. of amino acids in gp120/gp41	N-linked glycosylation sites in gp120/gp41	Net charge of V3	Comment
aBR-01	524/345	25/4	+3	
aBR-04	524/345	25/4	+3	Conserved His 252 replaced by Arg
aBL-01	517/345	23/5	+4	
bBR-01	514/345	29/4	+4	Conserved Cys 158 replaced by Arg
bBR-03	514/345	29/4	+4	Conserved Leu 578 replaced by Pro
bBL-05	512/345	28/4	+4	
cBR-06	515/346	32/5	+3	
cBR-07	515/346	31/4	+4	
cBL-02	509/200	28/3	+3	Premature stop codon in gp41
cBL-03	508/220	28/3	+3	Premature stop codon in gp41
dBR-02	504/345	23/4	+5	
dBR-07	504/345	23/4	+5	
dBL-04	504/345	23/4	+5	
89.6	508/345	25/5	+7	
ADA	508/346	26/5	+3	
HXB2	512/345	24/7	+9	

HXB2, and 24 to 28 for most clade B Envs in the Los Alamos database. The number of N-glycosylation sites in the gp41 proteins ranged from three to five. The net charge of the V3 loop influences HIV-1 tropism and coreceptor usage (3). The cloned Envs had V3 net charges between +3 and +5. V3 net charge was not associated with a particular pattern of coreceptor usage.

Analysis of the amino acid sequences revealed single amino acid changes potentially responsible for functional defects in three *env* clones (Table 3). The aBR-04 Env contains an arginine in place of a highly conserved histidine in C2 of gp120 (Fig. 1, position 252) (48). This region forms a beta structure and is sensitive to amino acid changes (22). The bBR-01 Env contains an arginine residue between V1 and V2 in place of a highly conserved cysteine (Fig. 1, position 158), a change predicted to result in misfolded V1/V2 loops. The bBR-03 Env has a highly conserved leucine in the heptad repeat 1 region of gp41 that is replaced by a proline (Fig. 1, position 578), which is likely to interfere with the helix formation of this region. Point mutations R252H, R158C, and P578L were introduced into the aBR-04, bBR-01, and bBR-03 Envs, respectively, by mutagenesis as described above by using the following primer pairs: aBR-04-H (5′-CAGCACAGTACAATGTACACATGG AATTAGGCCAGTAG-3′ and 5′-CTACTGGCCTAATTCC ATGTGTACATTGTACTGTGCTG-3′), bBR-01-C (5′-GGA GAAAGGAGAAATAAAAAACTGCAGTTTCAATATCA CGGCAAGC-3′ and 5′-GCTTGCGGTGATATTGAAACT GCAGTTTTTTATTTCTCCTTTCTCC-3′), and bBR-03-L (5′-GTCTGGGGCATCAAGCAGCTGCAGGCAAGAGTC CTGGC-3′ and 5′-GCCAGGACTCTTGCCTGCAGCTGCT TGATGCCCCAGAC-3′). 293T cells were transfected with the wild-type and mutant Envs, and cell lysates were analyzed by Western blotting. Impaired processing of the aBR-04, bBR-01, and bBR-03 gp160 proteins was restored by introduction of these point mutations (data not shown). However, processing of bBR-01-C was still inefficient compared to that for aBR-04-H and bBR-03-L. The single amino acid changes introduced into aBR-04-H, bBR-01-C, and b-BR-03-L restored Env function in syncytium and infection assays (Table 2). These results suggest that single amino acid changes at positions 158, 252, and 578 of gp160 can render Env proteins misfolded and thereby nonfunctional and highlight the critical importance of these amino acids for functional integrity.

Sensitivity to CCR5 and CXCR4 inhibitors. The sensitivity of the Envs to CCR5 and CXCR4 small molecule inhibitors was first tested by infecting U87 cells expressing CD4, CXCR4, and CCR5 in the presence of AMD3100, an inhibitor of CXCR4 (6, 37), and TAK-779, an inhibitor of CCR5 (2). TAK-779, but not AMD3100, inhibited entry mediated by the aBR-01, aBR-04-H, and bBR-03-L Envs (data not shown). In contrast, AMD3100, but not TAK-779, inhibited entry mediated by the aBL-01 Env. Neither TAK-779 nor AMD3100 alone had any significant effect on entry mediated by the dual-tropic dBR-02 and dBR-07 Envs. We then examined the effects of coreceptor-specific antibodies and inhibitors on virus replication in primary brain cultures. NL4-3 viruses containing the aBR-01, aBL-01, dBR-02, or dBR-07 Envs or the 89.6, ADA, or SG3 control viruses (16, 17, 30) were used to infect primary brain cultures in the absence or presence of CCR5 and CXCR4 monoclonal antibodies (2D7 and 12G5, respectively; Pharmingen), TAK-779, and/or AMD3100 (Fig. 3). 12G5 or AMD3100 reduced replication of the aBR-01, dBR-02, and dBR-07 viruses by approximately 50%. 2D7 or TAK-779 reduced replication of the aBR-01, dBR-02, and dBR-07 viruses by approximately 70 to 80%. Replication of the aBL-01 virus was strongly inhibited by CXCR4 or CCR5 inhibitors, probably due to the low levels of virus replication. Combinations of 12G5 and 2D7 or of AMD3100 and TAK-779 completely abolished infection with ADA, 89.6, and SG3, whereas infection with the aBR-01, dBR-02, and dBR-07 viruses was reduced by 90 to 95%. Thus, CCR5 is the principal coreceptor used by the aBR-01, dBR-02, and dBR-07 viruses for entry into microglia. CXCR4 was also used for entry into microglia but at lower efficiency than CCR5.

Sensitivity to neutralizing antibodies. Viruses pseudotyped with six primary Envs were tested for their sensitivity to neutralization by the F105 and 17b monoclonal antibodies (Fig. 4). Viruses pseudotyped with the HXB2, ADA, and 89.6 Envs were used as controls. F105 reduced the infectivity of HXB2 by 97%, whereas ADA and 89.6 were less sensitive to F105 neutralization, as described previously (40). F105 reduced the infectivity of viruses with the bBR-03-L, dBR-02, and dBR-07

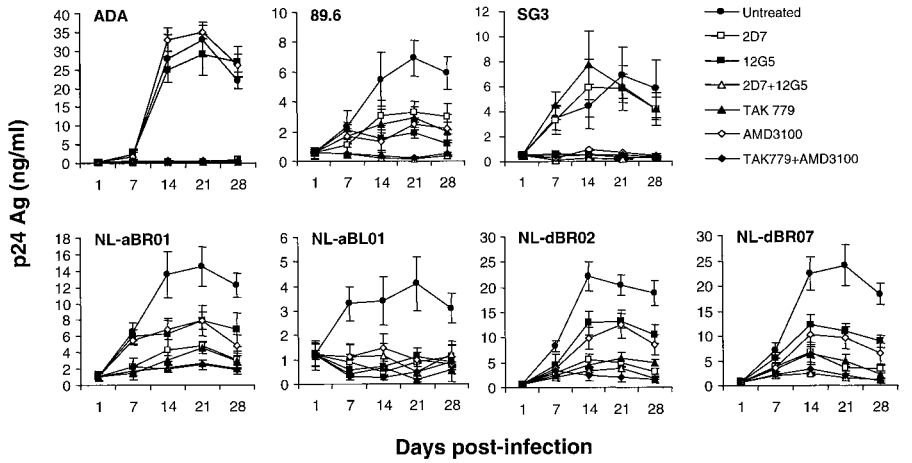


FIG. 3. Effects of CCR5 and CXCR4 antibodies and inhibitors on virus replication in primary brain cultures. Chimeric NL4-3 viruses containing the aBR-01, aBL-01, dBR-02, or dBR-07 Envs or the 89.6, ADA, or SG3 control viruses were used to infect primary brain cultures in the absence or presence of 10 μg of CCR5 and CXCR4 monoclonal antibodies (2D7 and 12G5, respectively) per ml, 100 nM TAK-770, and/or 1.2 μM AMD3100, as indicated. HIV-1 replication was monitored by quantitating HIV-1 p24 in culture supernatants. The values represent the means of the results of two experiments (mean ± standard deviation, $n = 2$).

Envs by 22 to 37%. In contrast, viruses containing Envs from patient A showed a modest enhancement of infectivity when the target cells expressed CCR5 (Fig. 4). A concentration of 100 μg of F105/ml was required to reduce the infectivity of viruses pseudotyped with aBL-01 by 62% (data not shown), whereas the same concentration abolished infection with HXB2 and 89.6 (data not shown). 17b reduced the infectivity of viruses pseudotyped with the primary Envs by 22 to 98% (Fig. 4). HXB2 was more sensitive than 89.6 and ADA to neutralization with 17b, a result similar to that obtained with F105. In contrast to F105, 17b did not exhibit any enhancing effect. These findings are consistent with those of previous studies which showed that primary Envs are much more resistant to neutralizing antibodies than lab-adapted Envs (26, 40) and indicate that brain-derived Envs exhibit heterogeneous sensitivity to neutralizing antibodies.

Conclusions. In this study, 37 full-length HIV-1 *env* genes were cloned directly from brain biopsy and blood samples from four patients with AIDS. Phylogenetic analysis showed distinct clustering of brain relative to blood *env* sequences, indicating tissue-specific compartmentalization of the virus. However, no brain-specific signature sequence was identified. Furthermore, there were no significant differences in the numbers or positions of N-linked glycosylation sites between brain and blood *env* sequences. The patterns of coreceptor usage were heterogeneous, with no clear distinction between brain and blood *env* clones. Nine *env* clones principally used CCR5, one principally used CXCR4, and two used CCR5 and CXCR4 equally well in cell-to-cell fusion assays. Eight *env* clones could also use alternative coreceptors (i.e., CCR3, CCR8, GPR15, STRL33, Apj, and GPR1) in fusion assays. We found a higher frequency of viruses that could use alternative coreceptors than was de-

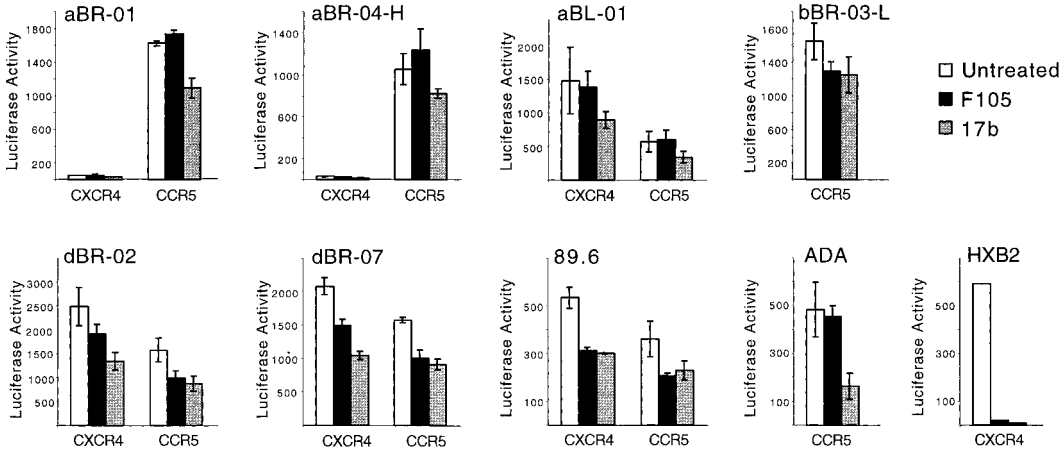


FIG. 4. Virus neutralization assays. Luciferase reporter viruses pseudotyped with aBR-01, aBR-04-H, aBL-01, bBR-03-L, dBR-02, dBR-07, 89.6, ADA, or HXB2 Envs were preincubated with 30 μg of 17b or F105 antibodies or medium without antibodies per ml prior to infection of U87 cells transfected with CD4 and CXCR4 or CCR5. Luciferase activity was measured 48 h postinfection. The values represent the means of the results of two experiments (mean ± standard deviation, $n = 2$).

scribed in previous studies (16, 49), which may reflect the high levels of coreceptor expression obtained in our assay system (16) or functional differences between Envs cloned from uncultured tissues versus those cloned from passaged virus isolates. Most Envs from the brain principally used CCR5, a finding consistent with those of previous studies (1, 16, 18, 38). However, we also cloned dual-tropic Envs from brain and found that such variants can be highly cytopathic for CNS cells, implying that R5X4 variants contribute to HIV-1 encephalitis and CNS injury in a subset of AIDS patients. These results contrast with those of previous reports, which suggested that HIV-1 viruses in brain are non-syncytium-inducing and macrophage-tropic mainly on the basis of V3 net charge (21). In fact, we found that V3 net charge did not predict coreceptor usage, a result consistent with those of other recent studies (16, 39).

The role of alternative coreceptors in HIV-1 entry and pathogenicity in the CNS remains unclear. Four brain-derived Envs used CCR3, STRL33 and/or GPR15 reasonably well for virus entry into transfected cells. CCR3 is expressed on microglia (18, 38), and STRL33 is expressed in the brain or on neurally derived cell lines. GPR15 is expressed in spleen, colon, and lymphoid tissue but, to our knowledge, has not been detected in the CNS. Despite the ability to use alternative coreceptors, aBR-01, dBR-02, and dBR-07 principally used CCR5 and CXCR4 for entry into microglia, a conclusion based on the observation that the combination of TAK-779 and AMD3100 inhibited replication of these viruses in microglia by 90 to 95%. Further studies are required to demonstrate whether the low level of residual virus replication reflects a low level of virus entry mediated by CCR3 (18) or another alternative coreceptor expressed on microglia.

Brain- and blood-derived Envs from patients B and C contain 28 to 32 N-linked glycosylation sites in gp120, compared to around 23 to 26 in lab strains and well-characterized primary isolates (e.g., HXB2, SF-2, ADA, YU-2, JRFL, JRCSF, BaL, and 89.6) and 24 to 28 for most clade B Envs (29). Furthermore, two Envs from patient C contain 35 and 37 N-glycosylation sites in gp160 compared to 25 to 34 in 148 subtype B HIV-1 Envs in the HIV sequence database (P. Rose and B. Korber, unpublished observation). The bBR-01-C and bBR-03-L Envs, which have 29 N-glycosylation sites in gp120, were functional in fusion and infection assays, while five others that have 28 to 32 were functional only in fusion assays. Competition between glycosylation sites and subsequent misfolding might underlie the inefficient processing of some Envs from patients B and C, since misfolding might occur when glycosylation sites are located in close proximity to each other and steric restrictions prevent their simultaneous usage. Short-term passage of HIV-1 can select for loss of N-glycosylation sites (25), which might explain why our study identified Envs that contain additional N-glycans compared to most gp160 proteins in the HIV sequence database (29). The existence of HIV-1 Env variants that are glycosylated more extensively than lab strains and well-characterized primary isolates should be considered during future efforts to develop vaccines and neutralizing antibodies, since the presence of additional N-glycans can shield neutralizing epitopes (22, 26, 45, 47).

Primary HIV-1 viruses are difficult to neutralize due to the relative inaccessibility of neutralizing antibody epitopes on the surfaces of the Envs (27, 40). The brain-derived Envs in our study were relatively resistant to neutralization by F105 and 17b antibodies. Resistance to F105 neutralization was variable, with Envs from patient A demonstrating less sensitivity to neutralization than Envs from patient D. In fact, slight enhancement of infection was observed for viruses with Envs from patient A, a finding consistent with that from a previous report which demonstrated F105-mediated enhancement of infection by ADA and YU-2 (40).

In summary, our studies suggest that HIV-1 variants in brain cannot be distinguished from those in blood on the basis of coreceptor usage or the number or positions of N glycosylation sites, indicating that other properties of the envelope glycoproteins underlie neurotropism. Elucidating the relationship between the genetic evolution of HIV-1 in specific tissue sites and the biological properties of the viral Envs may lead to a better understanding of adaptive changes that occur during viral evolution and the impact of these changes on disease pathogenesis and immune control.

Nucleotide sequence accession numbers. *env* sequences were submitted to GenBank under accession numbers AY124970 through AY124982 and AY159651 through AY159674.

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