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Isolation and Characterization of Human Immunodeficiency Virus Type 1 Variants Infectious to Brain-Derived Cells: Detection of Common Point Mutations in the V3 Region of the *env* Gene of the Variants

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T-cell-line-tropic human immunodeficiency virus type 1 cannot infect CD4-positive, brain-derived cells. We isolated several new variants that readily infected brain-derived cells. Mutation of proline to serine, to alanine, or to threonine in the well-conserved GPGR sequence in the V3 region of the envelope glycoprotein was found in all these variants. This indicates the importance of amino acid sequences at the tip of the V3 region for brain cell tropism of human immunodeficiency virus type 1.

Most cells that are susceptible to human immunodeficiency virus type 1 (HIV-1) express CD4 molecules on their surfaces (7, 14, 21, 31). However, not all cells expressing CD4 molecules are infected by various HIV-1 isolates (4, 5, 19). T-cell-line-tropic HIV-1 and macrophage-tropic HIV-1 have been well documented (12, 25). T-cell-line-tropic viruses scarcely infect macrophages, while macrophage-tropic viruses cannot infect T-cell-line cells. According to analyses of the viral genome of these viruses, this cell tropism is mainly determined by the third variable region (V3 region) of the viral envelope glycoprotein gp120 (12, 33, 39). There is also a report that T-cell-line-tropic HIV-1 cannot plated CD4-positive cells derived from the brain or skin (4). Nevertheless, we previously isolated an HIV-1 variant that infected CD4-positive brain cells and showed that a single point mutation at the proline of the glycine-proline-glycine-arginine (GPGR) sequence in the V3 region of gp120 was responsible for this brain cell tropism (35). So far, only one brain-cell-tropic (variant) virus from a T-cell-line-tropic (wild-type) HIV-1, i.e., HIV-1[GUN-1/WT], has been isolated (35). To determine whether HIV-1[GUN-1] is a special strain from which a variant HIV-1 could be isolated, we tried to isolate new variants from other HIV-1 strains. We also tried to isolate new HIV-1[GUN-1] variants from molecularly or biologically homogeneous wild-type virus to investigate whether there are common changes in the amino acid sequences of the V3 regions in variants derived from HIV-1[GUN-1].

The human T-cell lines MT-4 (24), MOLT-4 (22) and M8166 (a subclone of C8166 cell line) (30) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. U-87 MG (38) and U-251 MG (3) human glioblastoma cell lines and the U-87/CD4 cell line stably expressing the human CD4 gene (5) were maintained in Eagle's minimum essential medium (Nissui, Tokyo, Japan). The human fibroblast-like cell strain BT-3, derived from a human meningioma obtained during surgery, was cultured as described previously (35). The expressions of CD4 mRNA and CD4 antigen by BT-3 cells were confirmed by Northern (RNA) blot analysis and immunoprecipitation (data not shown). The HIV-1 strains GUN-1/WT, GUN-1/V, GUN-2, GUN-3, GUN-4, GUN-5, and GUN-6 were isolated as described elsewhere (32, 35, 36). GUN-7, GUN-8, and GUN-9 strains were isolated from Japanese hemophiliacs by cocultivation of their peripheral blood lymphocytes with MT-4 cells. The GUN-1 substrains GUN-1-1, GUN-1-2, and GUN-1-3 were plaque purified twice from HIV-1[GUN-1/WT] with MT-4 cells as described by Harada et al. (8). Previously we reported the construction of an infectious HIV-1[GUN-1] DNA clone (35). This DNA clone was transfected into MT-4 cells, and the recombinant HIV-1 recovered was named the GUN-1rWT strain. Human T-cell lymphotropic virus type III_B (HTLV-III_B) (28) was propagated in MOLT-4 cells, while HIV-1[SF-2] (18) and LAV-1 (2) were propagated in H9 cells (28).

HIV-1 variants that infected brain-derived cells were isolated by cocultivation of U-87/CD4 cells with MT-4 cells infected with wild-type HIV-1. MT-4 cells were infected with one of nine HIV-1[GUN] strains, HTLV-III_B, HIV-1[SF-2], or LAV-1. For GUN strains other than GUN-1, MT-4 cells were infected with isolates that had been stored at −80°C soon after virus isolation. MT-4 cells were also infected with various substrains derived from HIV-1[GUN-1]. When about half of the MT-4 cells became positive for HIV-1 antigens, they were overlaid on U-87/CD4 cells and the U-87/CD4 cells were passaged serially. During cultivation for 1 to 2 months, all MT-4 cells disappeared because of cytolysis and U-87/CD4 cells were monitored several times for expression of HIV-1 antigens by the indirect immunofluorescence assay (IFA). The culture supernatants of cells expressing these antigens were harvested and further inoculated onto new U-87/CD4 cells. Culture supernatants containing HIV-1 highly infectious to U-87/CD4 cells were stored frozen.

We isolated three new variants named GUN-1-1/V, GUN-1-2/V, and GUN-1-3/V from plaque-purified GUN-1 substrains GUN-1-1/WT, GUN-1-2/WT, and GUN-1-3/WT, respectively. The GUN-1rWT/V strain was also a newly isolated variant derived from the infectious DNA clone GUN-1rWT. In addition, we isolated two new variants named GUN-4/V and GUN-7/V. Although we isolated several variants from GUN-1 strains, we failed in repeated attempts to isolate variants from the HTLV-III_B, HIV-1[SF-2], or LAV-1 strain.

The infectivities of all wild-type and variant HIV-1 strains

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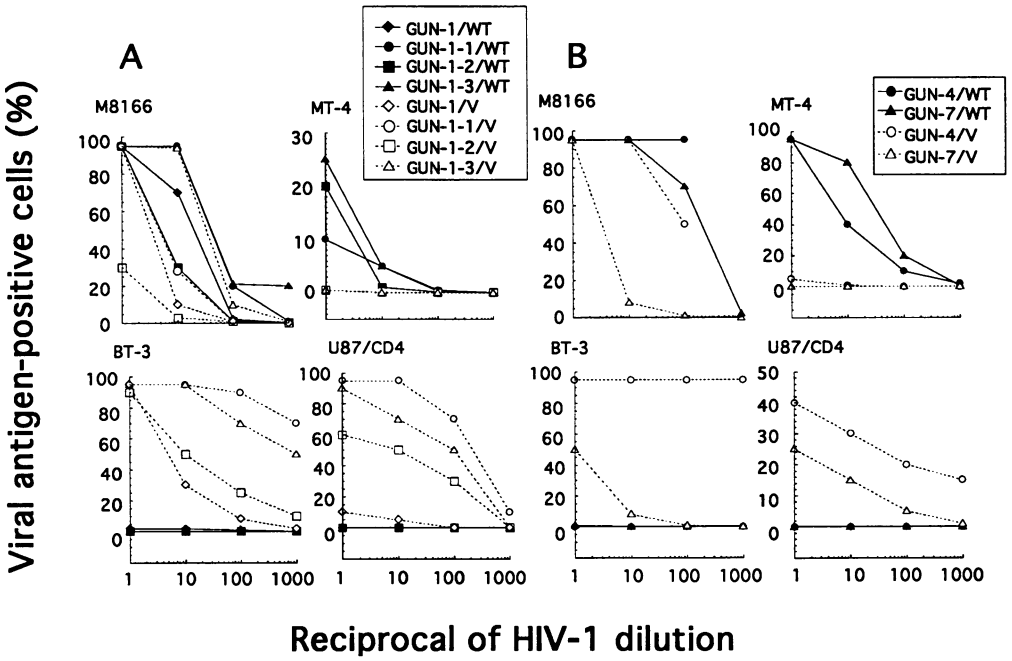

FIG. 1. Infection of human cells with wild types and variants related to three HIV-1[GUN] strains. GUN-1-related viruses (A) and GUN-4- or GUN-7-related viruses (B) were inoculated onto the T-cell lines M8166 and MT-4 and the brain-derived cell lines BT-3 and U-87/CD4. Expression of HIV-1 antigens was detected by IFA after cultivation for 4 days (M8166 and MT-4 cells) or 10 to 12 days (BT-3 and U-87/CD4 cells).

were compared by using HIV-1 stocks prepared in M8166 cells in order to exclude effects of the cells used for propagation on HIV-1 infectivity. U-87/CD4 and BT-3 cells were seeded into 12-well plates at 5×10^4 cells per well in 2 ml of culture medium. The next day, the medium was removed and viruses were inoculated after serial dilution: the lowest dilutions of inocula were adjusted to contain 1.5×10^5 cpm of reverse transcriptase activity. Four hours later, the inocula were removed, and the cells were washed once, maintained in culture medium, and passaged every 5 to 6 days. For infection of nonadherent M8166 or MT-4 cells, 5×10^4 to 7.5×10^4 cells were incubated with viruses for 2 h. Then the cells were washed once and maintained in RPMI 1640 medium containing 10% fetal calf serum. Reverse transcriptase activity in the culture medium was measured as described previously (11). Cells expressing HIV-1 antigens were detected by IFA.

M8166 or MT-4 cells infected with HIV-1 were subjected to IFA after cultivation for 4 days. Wild-type GUN-1 (Fig. 1A) and GUN-4 and GUN-7 (Fig. 1B) strains infected M8166 cells slightly more efficiently than the corresponding variants. This tendency was more clearly observed when MT-4 cells were infected with these viruses, i.e., the GUN-1, GUN-4, and GUN-7 variants hardly infected MT-4 cells. On the contrary, the variants efficiently plated brain-derived BT-3 or U-87/CD4 cells, as judged by examining these cells for expression of HIV-1 antigens after cultivation for 10 to 12 days (Fig. 1). Although new variants were isolated by using CD4-introduced U-87/CD4 cells, these variants also replicated well in naturally occurring, CD4-positive BT-3 cells. As expected, the wild-type viruses did not infect these cells. Small percentages of BT-3 cells, but not U-87/CD4 cells, became HIV-1 antigen positive after infection with some wild-type viruses, e.g., GUN-1/WT or HTLV-III_B, at low dilutions, but the ratio of infected cells did not increase even after cell passages, while the variants spread readily among the brain-derived cells after cell passages. CD4-negative, glioma-derived U-87 MG or U-251 MG cells were not infected with either the variants or the wild-type viruses (data not shown).

Because the V3 region has been reported to be mainly responsible for cell tropism of HIV-1 (13, 33, 35, 39), we focused attention on the amino acid sequences in and around the V3 regions of these variants. MT-4 cells infected with the wild-type viruses and U-87/CD4 cells infected with the variants after cocultivation or cell-free infection were used as sources of viral DNA. High-molecular-weight DNA from the HIV-1-infected cells was amplified by the nested PCR (29) using two primer pairs around the V3 region. The first PCR was carried out with oligonucleotides 670N (5′-GTAGTGTCAACCC AACTGCT-3′) and P4C (5′-GAAAAATTCCCCTCCAC-3′) corresponding to positions 660 to 679 and 1043 to 1027, respectively, in HIV-1[GUN-1] (35). The second PCR was carried out with 1 μl of the first PCR product and with oligonucleotides P2N (5′-AATGGCAGTCTAGCAGAAGA AGAG-3′) (positions 684 to 706) and P3C (5′-TTCTGGG TCCCCTCCTGAGGA-3′) (positions 1004 to 984). The expected length of the PCR product was 320 bp. PCR products were cloned into T vectors (20) constructed from pGEM5Zf(+) (Promega, Madison, Wis.) by using a ligation kit (Takara Shuzo, Kyoto, Japan) and transfected into *Escherichia coli*. PCR products in pGEM5Zf(+) were sequenced in an automated sequencer (model 373A sequencer; Applied Biosystems, Foster City, Calif.).

The amino acid sequences deduced from the DNA sequences of the V3 loops of the HIV-1[GUN-1] substrains are shown in Fig. 2A. Three plasmid clones containing the variant GUN-1rWT/V fragments, which had been derived from infectious DNA clone HIV-1[GUN-1rWT], were sequenced. For the plaque-purified substrains GUN-1-1, GUN-1-2, and GUN-1-3, one or two plasmid clones containing the wild-type HIV-1 fragments and three or four plasmid clones containing the

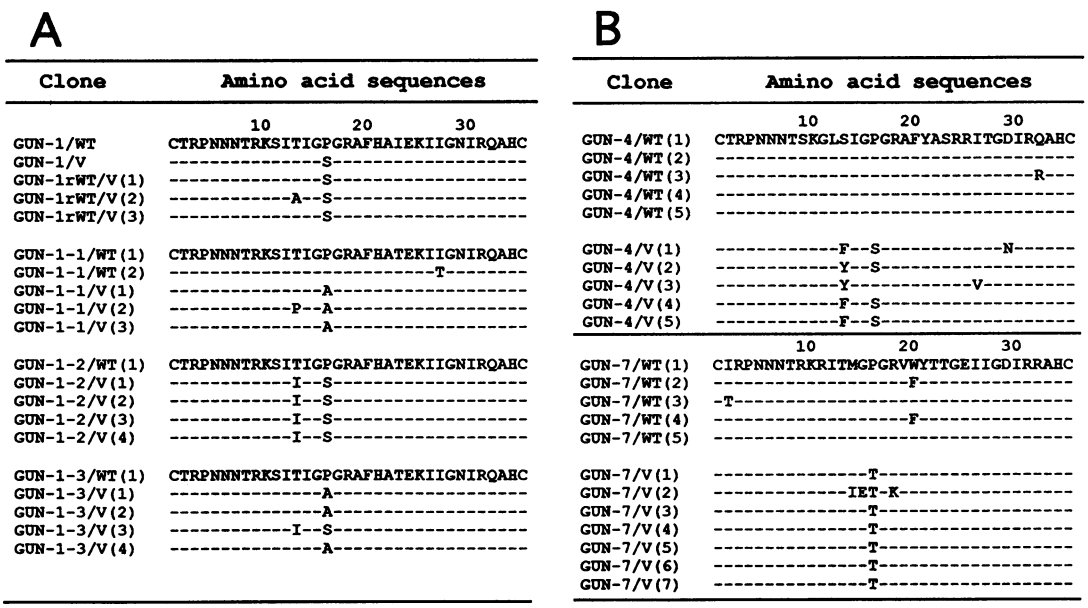

FIG. 2. Comparison of the amino acid sequences of the V3 loops of wild-type GUN-1, GUN-4, and GUN-7 strains and their variants. The amino acid sequences were deduced from DNA sequence data. The numbers of the plasmid clones used for DNA sequencing of each strain are shown in parentheses. Dashes indicate that the deduced amino acid was the same as that in the wild-type clone of the respective strain. (A) GUN-1-related strains; (B) GUN-4 and GUN-7 strains.

variant fragments were sequenced. The amino acid sequences of the wild-type GUN-1 strains were well conserved. Comparison of the amino acid sequences of the wild types and their corresponding variants showed that in all cases the amino acid at position 16 of the V3 loop was changed: the 16th amino acid of the V3 loop of all three wild-type substrains, GUN-1-1/WT, GUN-1-2/WT, and GUN-1-3/WT, was proline (P), whereas P was replaced by serine (S) or alanine (A) in the variants. Threonine (T) at position 13 of GUN-1-2/WT was replaced by isoleucine (I) in GUN-1-2/V, and one plasmid clone in each group of the variants contained A, P, or I at this position.

Next the plasmid clones containing the wild-type and the variant DNA fragments of HIV-1[GUN-4] were sequenced, and their deduced amino acid sequences are shown in Fig. 2B. Comparison of their sequences showed that P at position 16 of the V3 loop of the wild-type GUN-4/WT was again replaced by S in all four variants except plasmid clone no. 3. S at position 13 was replaced by phenylalanine (F) or tyrosine (Y) in all plasmid clones containing HIV-1[GUN-4/V]. The amino acid sequences of GUN-7-related strains were also deduced from the DNA sequences of the plasmid clones containing the wild-type GUN-7 fragments and the variant fragments (Fig. 2B). P at position 16 of the V3 loop of the wild type was replaced by T in all the variants. Thus, all HIV-1 variants derived from the GUN-1, GUN-4, or GUN-7 strain showed changes of P in the GPGR sequence. These findings suggested that an amino acid change at position 16 is necessary for formation of variants that can infect brain-derived, CD4-positive cells in vitro.

The substitution of P in the GPGR sequence was caused by a mutation of the first nucleotide of the proline codon CCA; the resulting codons, TCA, GCA, and ACA, code for serine, alanine, and threonine, respectively. Two plasmid clones of HIV-1[GUN-4/WT] and one clone of HIV-1[GUN-7/WT] which are not listed in Fig. 2B contained defective viral sequences, because they had a stop codon in or around the V3 sequence. One plasmid clone derived from U-87/CD4 cells infected with GUN-4/V had the GPGR sequence. Because the cellular DNA was extracted from U-87/CD4 cells cocultivated with MT-4 cells that had been infected with HIV-1[GUN-4/WT], pseudotype viruses having the envelope of variants or HTLV-I might have entered U-87/CD4 cells (15). Another possibility is that wild-type viruses might have plated the cells, because wild-type HIV-1 has been reported to enter CD4-negative glioma cells (9).

It is possible that brain-cell-tropic variants might have been isolated only from HIV-1 strains closely related to each other, because Japanese hemophiliacs have been infected with HIV-1 by blood products prepared in the United States. We examined this possibility by calculating the homologies of the DNA sequences of the GUN-1, GUN-4, and GUN-7 strains (Table 1). Comparison of the DNA sequences of the V3 loops showed that the homology rates of the different strains were less than 86%, indicating that GUN-1, GUN-4, and GUN-7 are clearly different HIV-1 strains. Thus, the isolated variants were not specifically associated with strains related to the GUN-1 strain. Phylogenic analyses of GUN strains (32) also showed that HIV-1[GUN-1/WT] is not very closely related to HIV-1[GUN-4/WT]. The homology rates of the wild types and variants of the same strains were more than 97%. We could not isolate any variants of the HTLV-III_B, LAV-1, or HIV-1[SF-2] strain, although we isolated several GUN-1 variants. GUN-4 and GUN-7 variants were isolated after a few trials. Because the mutation rate of HIV-1 is expected to be very high, variants should have been isolated more easily from other HIV-1 strains such as HTLV-III_B. This suggests that not only a single point mutation but also additional genetic changes may be required for most HIV-1 strains to become able to infect brain-derived cells.

To confirm that the cell tropism of newly isolated variants is determined by the DNA sequence in and around the V3 loop, we prepared chimeric HIV-1 containing the fragment of the

TABLE 1. DNA sequence homology of V3 loops of virus strains

HIV-1 strain and type	% DNA sequence homology of[a]: GUN-1 type WT	GUN-1 type V	GUN-4 type WT	GUN-4 type V	GUN-7 type WT	GUN-7 type V
GUN-1/WT	100	99	85	84	86	85
GUN-1/V		100	84	83	85	84
GUN-4/WT			100	97	82	81
GUN-4/V				100	80	80
GUN-7/WT					100	99
GUN-7/V						100

[a] Percent homology = (number of identical bases/number of total bases) × 100. GUN-1/WT, GUN-1/V, GUN-4/WT(1), GUN-4/V(1), GUN-7/WT(1), and GUN-7/V(1) were used for calculations because comparison of the DNA sequences in plasmid clones containing these types of HIV-1 showed the least variation.

V3-to-V5 region of GUN-4/WT or GUN-4/V on the backbone of GUN-1/WT. For this, the *Bgl*II fragment containing the V3-to-V5 region of the envelope gene of the GUN-4/WT or GUN-4/V strain was produced by PCR with primers Bgl-3 and Bgl-4. Two pairs of primers were used for nested PCR: the outer primers were P2N, described above, and 1590C (5′-C TGTGAGTTGCAACAGATGC-3′) (positions 1596 to 1577), and the inner primers were Bgl-3 (5′-TAATTAGATCTGA CAATTTCACGGACAA-3′) (positions 712 to 739) and Bgl-4 (5′-ATATCTCCTCCTCCAGGTCTGAAGATCTC-3′) (positions 1309 to 1281). The expected length of this PCR product was 597 bp, encompassing the V3-to-V5 region. The sequences of the inner primers were modified to contain a *Bgl*II site. The PCR products were digested with *Bgl*II and cloned into pBGL. pBGL was made of pUC118 (37) containing the HIV-1 DNA fragment between two *Pst*I sites of GW32, which has been named the Ps-b fragment (35).

Two GUN-1/WT DNA fragments (GUN-4rWT and GUN-4rV) containing the chimeric Ps-b fragments derived from GUN-4/WT and GUN-4/V, respectively, were transfected into M8166 cells by the modified DEAE-dextran method (1). Briefly, M8166 cells (10^6) were resuspended in a mixture of 200 μl of Tris-EDTA buffer containing DNA and 200 μl of DEAE-dextran (1 mg/ml). After transfection, the cells were seeded into 60-mm-diameter dishes, and virus replication in the transfectants was monitored every 3 or 4 days by checking for syncytium formation and by IFA. When more than 90% of the cells became HIV-1 antigen positive, the culture supernatants of the transfectants were harvested. The chimeric GUN-4rV virus carrying the *Bgl*II fragment derived from GUN-4/V could infect U-87/CD4 cells and spread among them after serial passages, whereas the chimeric GUN-4rWT virus could not infect these cells (Fig. 3B). Both chimeric viruses spread more slowly among M8166 cells and induced smaller syncytia in M8166 cells than the parental HIV-1[GUN-1] or HIV-1[GUN-4] strain (data not shown).

The sequences of the *Bgl*II fragments of the GUN-4/WT and GUN-4/V strains used to make the chimeric viruses are shown in Fig. 3A. Several amino acid changes were observed in the V3-to-V5 region. However, analyses of the amino acid sequences of all GUN-4-related clones (Fig. 2B and data not shown) revealed that the common changes of amino acid sequences in the wild types and variants of GUN-4 were all in the V3 region and the putative CD4-binding site (17, 26). These changes are mainly responsible for the brain cell tropism

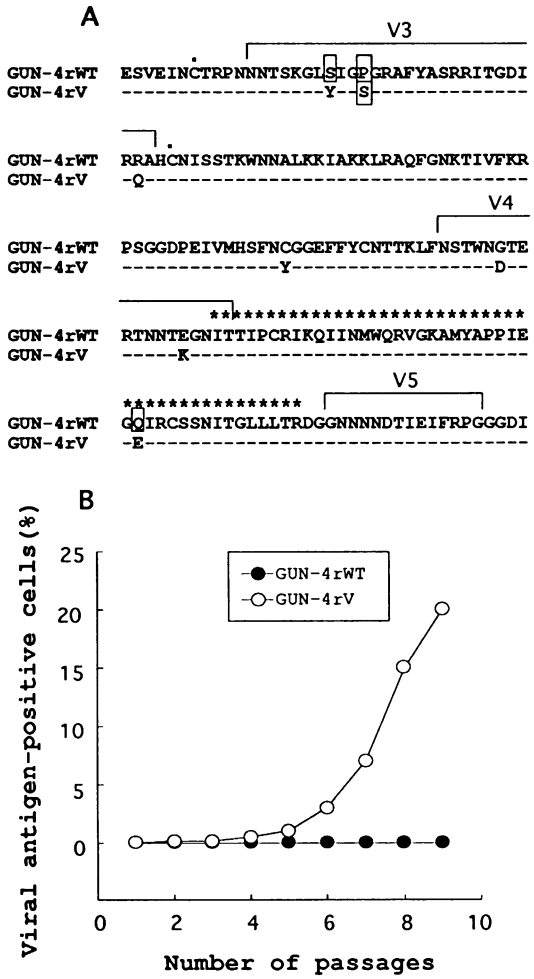

FIG. 3. Comparison of infectious clones containing the *Bgl*II fragments of GUN-4 sequences. (A) Differences in the amino acid sequences of *Bgl*II fragments in GUN-4/WT and GUN-4/V. Asterisks indicate the putative CD4-binding site. Boxes on the GUN-4rWT sequence indicate amino acids that were commonly substituted in all GUN-4 variants. The box on the GUN-4rV sequence indicates the substituted amino acid, i.e., S, that was the same among all variants. (B) Infectivities in U-87/CD4 cells of two infectious clones, GUN-4rWT and GUN-4rV, containing the *Bgl*II fragments of GUN-4/WT and GUN-4/V, respectively. The U-87/CD4 cells were passaged every 5 to 6 days. Expression of viral antigen was detected by IFA.

of the GUN-4 variants, although no changes of amino acids in the CD4-binding region were detected in the GUN-1 variants (data not shown).

As discussed above, the V3 regions of HIV-1 strains that could infect CD4-positive, brain-derived cells had the GSGR, GAGR, or GTGR sequence instead of the GPGR sequence. Furthermore, preliminary site-directed mutagenesis experiments using GUN-1rWT showed that mutants having S, A, or T in place of P in the GPGR sequence were highly infectious for BT-3 and U-87/CD4 cells (unpublished data), indicating that a change of P to S, A, or T in the V3 region is necessary and sufficient for HIV-1[GUN-1] to be able to infect CD4-positive, brain-derived cells. Thus, the mutation from P to T found in GUN-7/V also plays a role in the brain cell tropism of this variant. Page et al. (27) reported that the substitution of S or A for P in the GPGR sequence does not affect the ability of

HIV-1 to bind to the CD4 receptor. Therefore, the change of P in the GPGR sequence may affect a step of HIV-1 infection after CD4 binding, probably the fusion process. There are also reports that a mutation from P to S or A in the GPGR sequence altered the infectivity of T- or B-cell lines by HIV-1 strains (13, 27).

The amino acid sequence of GPGR in the V3 loop had been reported to be conserved in most HIV-1 strains and is predicted to form a type II β-turn (16). Furthermore, LaRosa et al. (16) detected a GAGR or GSGR sequence in the V3 region in about 1% of peripheral blood mononuclear cell samples isolated from 245 patients. Thus, HIV-1 that may show brain cell tropism under our assay conditions might exist in HIV-1-infected persons. In our experiment new HIV-1 variants were isolated by using U-87/CD4 cells originally derived from a human glioma. These variants plated BT-3 cells as well as or better than they did U-87/CD4 cells (Fig. 1). BT-3 cells were naturally occurring cells isolated from a human meningioma, while U-87/CD4 cells were artificial cells because CD4-expressing retrovirus had been introduced. Still, both cells showed similar susceptibilities to HIV-1 variants. It is important to determine the frequencies of HIV-1 with the GSGR, GAGR, or GTGR sequence in the V3 region in brain tissues from HIV-1-infected persons to evaluate the pathological significance of these variants.

The V3 loop has been shown to contain a potential cleavage site for a cellular protease (6, 10, 34). This cleavage is supposed to be important for the fusion process. If the cell tropism of HIV-1 is determined by the fusion process, our results suggest that the protease expressed in T-cell lines may not recognize V3 amino acid sequences containing GSGR, GAGR, or GTGR well, while a protease of brain-derived cells may react with these sequences but not with the GPGR sequence. This kind of specificity as a substrate of an enzyme is likely because serine, threonine, and alanine are suggested to have similar biochemical characters (23). Consistent with this possibility, we found that even when small percentages of human T-cell lines were infected with variants, as shown in Fig. 1, they spread after serial cell passage, whereas wild-type HIV-1 did not spread secondarily in brain-derived cells, as shown in Fig. 3B, even when a small percentage of the cells was positive for HIV-1 antigen after initial infection. Therefore, in brain cells, restriction to the spread of the wild types is almost complete.

The CD4 molecule is the main receptor for HIV-1, but it is supposed that the second factor on the cell surface is necessary for HIV-1 infection (5). Cell tropism of HIV-1 is probably determined by a difference in this factor. Study of the brain cell tropism of HIV-1 may clarify mechanisms that also operate in infection of T cells or macrophages with HIV-1, and this kind of study may contribute to the development of a new strategy to control HIV-1 infection in vivo.

Nucleotide sequence accession numbers. The DNA sequences of the HIV-1 strains determined in this study have been deposited in GenBank under accession numbers D34590 to D34611.

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