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Sequences Regulating Tropism of Human Immunodeficiency Virus Type 1 for Brain Capillary Endothelial Cells Map to a Unique Region on the Viral Genome

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Two infectious molecular clones of human immunodeficiency virus type 1, NL4-3 and JR-CSF, differ in their abilities to productively infect human brain capillary endothelial (HBCE) cells. The phenotypes of recombinants between these two molecular strains were examined to identify viral sequences responsible for the difference in HBCE cell tropism between the two parental strains. Our results indicate that HBCE cell tropism maps to a region that encompasses the C1 region of *env* and includes overlapping reading frames for the accessory genes *vpr*, *vpu*, *tat*, and *rev*. This region was unique for HBCE cell tropism and did not cosegregate with either macrophage or T-cell line tropism. However, several recombinant clones displayed dual tropism for both HBCE cells and macrophages. These endothelial cell- and macrophage-tropic strains may have a unique pathogenic advantage by entering the brain via HBCE cells and subsequently infecting microglial cells with high efficiency, leading to the induction of human immunodeficiency virus dementia.

Human immunodeficiency virus (HIV) dementia is an important clinical syndrome occurring late in the course of AIDS progression in approximately 20% of patients (33, 40). Infectious HIV virions and/or viral protein antigens have been detected in the brain tissue of numerous patients with AIDS both with and without clinical neurological symptoms (24, 46, 53, 56). Thus, it is unclear why only a subset of patients develops primary clinical HIV-induced central nervous system (CNS) disease. At present little is known about how HIV enters the CNS or the pathogenic mechanisms involved in HIV-induced brain disease. Some evidence suggests that unique HIV strains might account for these clinical differences (44, 43). For example, virus strains differing in neuropathogenesis could vary in route of infection to the brain, types of brain cells infected, and neurotoxic potency of viral products (14, 51).

Different isolates of HIV show broad phenotypic variation including a differential capacity to replicate in certain cell types. HIV strain differences in cell tropism may also be important in induction of neuropathogenesis. Although all HIV strains appear to infect peripheral CD4-positive lymphocytes, HIV strains vary in their abilities to induce syncytia in these lymphocytes in vitro (50) and in their in vitro infectivities for macrophages (12, 22, 41, 47, 56), microglial cells (43, 45), CD4-positive HeLa cells (12), and immortalized leukemia T-cell lines (26). In most of these cell types the V3 region of the viral envelope appears to exert the main influence on infectivity, but in some cases other envelope regions (25, 55), as well as nonenvelope genes, including *vpr* (2, 3, 21), *vpu* (2), and *nef* (34), have been shown to influence tropism.

Evidence from several murine retroviral models indicates that infection of brain capillary endothelial cells is an important step in transfer of retroviruses from the blood to the brain parenchyma (20, 22, 49). Similarly, HIV has been detected in human brain capillary endothelial (HBCE) cells in the brains of patients with AIDS (55), and we have shown that HBCE cells can be productively infected by HIV in vitro (35). Alternatively, HIV-infected peripheral blood monocytes or lymphocytes may extravasate directly through the blood-brain barrier composed of endothelial cells and astrocytes. HIV-infected infiltrating cells have been observed surrounding brain blood vessels by several groups (15, 48, 56). In both of these situations, specific interactions between HBCE cells and HIV-infected cells may play a critical role.

HIV strains have also been shown to vary in tropism for HBCE cells (37). Surprisingly, while the blood-derived laboratory-adapted strain LAV (4) showed high infectivity for HBCE cells, brain-derived macrophage strains JR-CSF (27) and JR-FL (27) showed low infectivity (37). In the present study we examined HBCE cell infectivities of recombinant HIV clones derived from another laboratory-adapted, HBCE cell-tropic strain, NL4-3 (1), and the non-HBCE cell-tropic strain JR-CSF to determine the regions of the viral genome involved in HBCE cell tropism. The results indicate that sequences upstream of the V3-encoding region including *env*, *vpr*, *vpu*, *tat* and *rev* influence HBCE cell tropism. Furthermore, this tropism appears to be independent of the V3-induced effect on tropism for macrophages and microglial cells. The ability of HIV to infect HBCE cells may be an important factor determining the time and route of entry of HIV into the CNS.

MATERIALS AND METHODS

Cells. HBCE cells were isolated from the temporal lobe brain tissue of HIV-seronegative adults as previously described (35). HBCE cells were cultured in endothelial cell growth medium consisting of endothelial-SFM medium (GIBCO, Grand Island, N.Y.) supplemented with 10% human AB serum (Sigma Chemical Co., St. Louis, Mo.), 50 μg of endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, Mass.) per ml, and 40 μg of heparin (Sigma) per ml. The purity of endothelial cell cultures was confirmed by positive staining for endothelial cell-specific von Willebrand factor as previously described (35). For HIV infections, heparin was omitted from the culture medium.

Macrophages were isolated from the peripheral blood of HIV-seronegative

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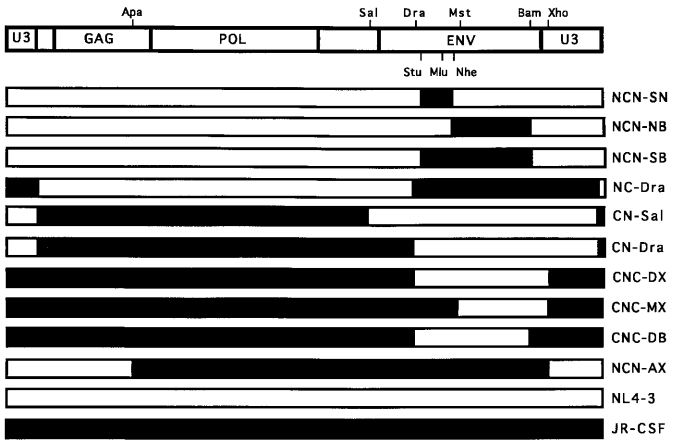

FIG. 1. Diagramatic representation of recombinant HIV constructs derived from the parental infectious molecular clones NL4-3 and JR-CSF. Open bars, sequences contributed by NL4-3; filled bars, sequences contributed by JR-CSF. The positions of these regions on the HIV genome and the restriction enzyme sites used to construct the recombinants are shown. Restriction enzyme sites correspond to the following positions in NL4-3: *Apa*I, 2006; *Sal*I, 5785; *Dra*III, 6591; *Stu*I, 6822; *Nhe*I, 7250; *Mst*II, 7305; *Bam*HI, 8465; *Xho*I, 8887. The *Mlu*I site at position 7121 is included to indicate the position of the V3 loop region (*Mlu*I to *Nhe*I) of gp120.

donors as previously described (36). Macrophages were cultured in macrophage growth medium consisting of 60% AIM V medium (GIBCO), 30% Iscove's medium (GIBCO), and 10% human AB serum.

HeLa $CD4^+$ clone 1022 cells (HeLa CD4 cells) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (11). HeLa CD4 cells were cultured in Dulbecco modified Eagle medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum (Gemini Bioproducts Inc., Calabasas, Calif.) and G418 (500 μg/ml).

Virus. HIV-1 strain LAV was derived from the culture supernatant of the persistently infected cell line HeLa/LAV (4). HIV-1 strain IIIMN was derived from the culture supernatant of the persistently infected cell line H9/HTLV-IIIMN NIH 1984 (18). Both cell lines were obtained through the AIDS Research and Reference Reagent Program. SF-2 (29), SF-162 (9), and JR-FL (27) were obtained as cell-free stocks through the AIDS Research and Reference Reagent Program and propagated on phytohemagglutinin-stimulated normal human peripheral blood mononuclear cell cultures. Parental HIV-1 strains NL4-3 (6) and JR-CSF (27) and recombinant virus constructs have been previously described (10). Recombinant viruses NCN-SN, NCN-NB, NCN-SB, NC-Dra, CN-Sal, CN-Dra, CNC-DX, CNC-MX, and NCN-AX were constructed from infectious molecular clones of JR-CSF and NL4-3 as previously described (10). Recombinant virus CNC-DB was constructed by a similar method. Figure 1 shows the regions of the viral genome that were contributed by each virus. Infectious HIV stocks were produced by transfection of recombinant and parental viral plasmids into phytohemagglutinin-stimulated peripheral blood mononuclear cells (10).

HIV infections. Low-passage-number endothelial cells were plated in 60-mm^3 Primaria (Becton Dickinson, Lincoln Park, N.J.) culture dishes and grown to 60% confluence prior to infection with HIV. Infections were performed by incubating HBCE cell monolayers with 1.5 ml of virus inoculum for 4 h at 37°C. Virus stocks were diluted in endothelial cell growth medium to obtain inocula with equivalent titers as determined on phytohemagglutinin stimulated-blasts (5×10^3 PFU/ml). After 4 h, HBCE cell monolayers were rinsed three times in phosphate-buffered saline (PBS) and once in endothelial cell growth medium to remove unadsorbed virus and recultured in fresh medium. The production of HIV was followed by measuring $p24^{gag}$ antigen levels in culture supernatants. For this purpose, medium was changed every 48 h and harvested culture supernatants were stored at −80°C until testing. Selected monolayers were processed for detection of intracellular p24 antigen by indirect immunofluorescent staining at intervals up to 2 weeks postinfection (p.i.).

Macrophages differentiated for 7 days in culture were infected with selected recombinant viruses as previously described (52). Infection was detected by immunofluorescent staining for p24 antigen at day 14 p.i. and by observation of cytopathic effects up to 3 weeks p.i.

HeLa CD4 cells were seeded into six-well culture dishes and grown to 50% confluence prior to infection. HeLa CD4 cells were infected with serial dilutions of selected recombinant viruses for 4 h using the protocol described for infection of HBCE cells. At day 4 p.i., monolayers were fixed in methanol and stained with 1% crystal violet to detect infection foci (51). The HIV specificity of foci was confirmed by immunofluorescent staining for p24 antigen.

HIV antigen assay. HIV p24 antigen levels in HBCE cell supernatants were determined by a commercial enzyme-linked immunosorbent assay (ELISA; Coulter Corporation, Hialeah, Fla.). Antigen levels (in picograms per milliliter) were calculated by using the Retrovirus Laboratory Management Program software (Dataworks Development Inc., Mountlake Terrace, Wash.).

Immunofluorescence. The presence of p24 antigen in HBCE cells or macrophages was detected by indirect immunofluorescence staining. Cell monolayers were fixed in 95% ethanol–5% glacial acetic acid for 10 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature, and blocked with 20% normal goat serum in PBS for 30 min at 37°C. Cells were incubated with a 1:100 dilution of a murine monoclonal anti-p24 antibody (Agmed Inc., Bedford, Mass.) or clone 183-H12-5C and then with a 1:100 dilution of a fluorescein-conjugated goat anti-mouse secondary antibody (Tago). Both incubations were performed for 75 min at 37°C. Cells were examined for p24 staining by fluorescence microscopy using a Nikon Optiphot inverted microscope.

RESULTS

HIV strains demonstrate differential tropism for HBCE cells. To determine whether HIV demonstrates differential tropism for HBCE cells, several different well-characterized strains were examined for growth in these cells. The HIV strains tested included LAV, IIIMN, NL4-3, and SF-2, which grow efficiently in T-cell lines, and macrophage-tropic strains SF162, JR-FL, and JR-CSF. As shown in Table 1, LAV, IIIMN, SF-2, and NL4-3 produced high p24 levels in HBCE cells at 5 and 10 days p.i. In contrast, JR-CSF demonstrated significantly lower p24 levels, suggesting minimal virus replication in HBCE cells, and p24 was not detected in supernatants from HBCE cells exposed to JR-FL or SF-162. Results obtained for supernatant p24 levels were confirmed by examination of intracellular p24 by fluorescence microscopy (Table 1). p24 antigen was readily detected in HBCE cell cultures infected with LAV,

TABLE 1. Infectivities of selected HIV isolates for HBCE cells

Isolate	Supernatant p24 (pg/ml)[a,b]		% $p24^+$ cells[b,c]
	Day 5 p.i.	Day 10 p.i.	
LAV	506	>739	20
IIIMN	>739	>739	20
SF-2	704	659	15
NL 4-3	576	461	10
JR-CSF	96	89	Not detected

[a] Levels of p24 antigen in 48-h HBCE cell culture supernatants were measured by a commercial ELISA.

[b] No p24 was detected for isolates JR-FL and SF-162.

[c] Intracellular p24 antigen was detected by immunofluorescent staining of fixed HBCE cell monolayers at day 10 p.i.

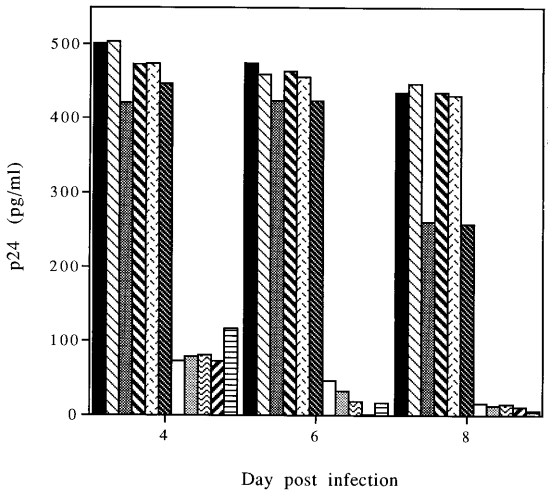

FIG. 2. Tropism of recombinant HIV strains for HBCE cells. HBCE cell culture supernatants were harvested at 48-h intervals on days 4, 6, and 8 p.i., and amounts of supernatant p24 were determined by ELISA. As a positive control, HBCE cells were infected with the HBCE cell-tropic strain IIIMN. HBCE cell-tropic viruses: IIIMN (■), NCN-SN (▧), NCN-NB (▩), NCN-SB (◥), NC-Dra (◱), and CN-Sal (◥); non-HBCE cell-tropic viruses: CN-Dra (□), CNC-DX (▤), CNC-MX (⊟), CNC-DB (◰), and NCN-AX (▭). The values are representative of four separate experiments.

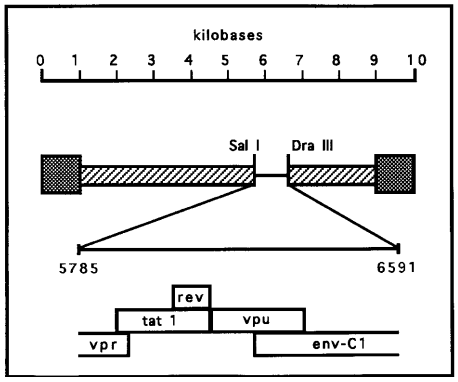

FIG. 3. Schematic of the genetic composition of the region of HIV clone NL4-3 delimited by the *Sal*I (position 5785) and *Dra*III (position 6591) restriction sites.

IIIMN, SF-2, and NL4-3 at day 10 p.i., while staining was not observed in cells exposed to JR-CSF, JR-FL, or SF-162. These results indicate that HIV strains demonstrate a differential capacity to infect HBCE cells.

Replication of NL4-3/JR-CSF recombinant viruses in HBCE cells. Since infectious molecular clones of NL4-3 and JR-CSF were available, the genetic basis for HIV growth in HBCE cells was investigated with recombinant clones. Recombinant infectious HIV clones were generated by exchanging different portions of the NL4-3 and JR-CSF genomes. The segments of the viral genome contributed by each virus are depicted in Fig. 1. Infectivity was determined by measurement of p24 levels in 48-h culture supernatants at sequential times p.i. (Fig. 2). Recombinant viruses were divided into two distinct groups on the basis of their abilities to infect HBCE cells. The viruses designated NCN-SN, NCN-NB, NCN-SB, NC-Dra, and CN-Sal demonstrated significant supernatant p24 levels in HBCE cell cultures. In contrast, infection of HBCE cells with viruses CN-Dra, CNC-DX, CNC-MX, CNC-DB, and NCN-AX resulted in background p24 levels. Relative infectivity was confirmed by immunofluorescent staining for intracellular p24 antigen. Positive staining for p24 was detected only in those cultures that produced positive ELISA results (data not shown).

The relative abilities of the recombinant constructs to infect HBCE cells were used to determine which regions of the NL4-3 genome conferred HBCE cell tropism. The only region of NL4-3 common to all five HBCE cell-tropic recombinants is an NL4-3 insert delimited by *Sal*I at position 5785 and *Dra*III at position 6591. This observation suggests that the sequence(s) that determines the HBCE cell tropism of NL4-3 is contained within the *Sal*I-to-*Dra*III region. The *Sal*I-to-*Dra*III region encodes the C1 region of env but also contains overlapping reading frames for the regulatory gene products vpr, vpu, rev, and tat (Fig. 3). A consistent feature of the five non-HBCE cell-tropic viruses was that they all contained JR-CSF sequences in the *Sal*I-to-*Dra*III region.

Four of the five viruses that infected HBCE cells were backbones of NL4-3 containing JR-CSF insert sequences (NCN-SN, NCN-NB, NCN-SB, and NC-Dra). These JR-CSF inserts contained most of the envelope sequence of JR-CSF (from *Dra*III at position 6591 to the U5 position) including the JR-CSF V3 loop domain. The ability of these viruses to infect HBCE cells indicated that envelope sequences downstream of the *Dra*III site at position 6591 do not define the HBCE cell-tropic phenotype of NL4-3. This observation was confirmed by the inability of a virus (CN-Dra) containing these NL4-3 downstream sequences in a JR-CSF backbone to infect HBCE cells. Additionally, the HBCE cell infectivity of the NC-Dra and CN-Sal viruses indicated that sequences upstream of the *Sal*I site at position 5785 could be contributed by either NL4-3 (NC-Dra) or JR-CSF (CN-Sal) without affecting HBCE cell tropism.

Endothelial cell tropism does not cosegregate with macrophage tropism. While most HIV isolates replicate efficiently in primary CD4-positive T cells, most macrophage-tropic strains demonstrate restricted growth in immortalized cell lines (12, 26). The tropism of the NL4-3/JR-CSF recombinants for macrophages and immortalized HeLa CD4 cells has been previously described (10). To directly examine whether growth in HBCE cells correlated with tropism for either of these cell types, we reexamined the ability of the HBCE cell-tropic NL4-3/JR-CSF recombinant clones to replicate in primary monocyte-derived macrophages and HeLa CD4 cells. Interestingly, only three of the HBCE cell-tropic recombinants, constructs NCN-SN, NCN-SB, and NC-Dra, were macrophage tropic, while the parental NL4-3 and constructs NCN-NB and CN-Sal replicated only in HeLa CD4 cells (Table 2). Macrophage tropism was dependent on the presence of the JR-CSF *Stu*I-*Nhe*I region which includes the gp120 V3 domain (*Mlu*I-*Nhe*I), while HeLa CD4 cell tropism was abolished by insertion of these JR-CSF sequences. Endothelial cell tropism, however, was not influenced by the presence of JR-CSF or NL4-3 sequences in this region. These observations suggest that HBCE cell infectivity does not cosegregate exclusively with the ability to infect either macrophages or HeLa CD4 cells and that molecular determinants of macrophage tropism do not influence HBCE cell tropism.

DISCUSSION

In this study, we demonstrate that different isolates of HIV display different capacities to infect HBCE cells. The availability of HBCE cell-tropic (NL4-3) and -nontropic (JR-CSF) viral

TABLE 2. Tropism of HBCE cell-tropic[a] HIV for macrophages and HeLa CD4 cells

Isolate	Tropism	
	Macrophage[b]	HeLa CD4[c]
NL4-3	No	Yes
NCN-SN	Yes	No
NCN-NB	No	Yes
NCN-SB	Yes	No
NC-Dra	Yes	No
CN-Sal	No	Yes

[a] As determined by p24 antigen capture ELISA on HBCE cell culture supernatants.

[b] Macrophage tropism was determined by immunofluorescent staining for HIV p24 at day 14 p.i. For macrophage-tropic viruses, >10% HIV p24-positive cells were routinely observed. For viruses that did not infect macrophages, no staining was observed.

[c] HeLa CD4 cell tropism was assessed by scoring adherent cell syncytia at day 4 p.i. For HeLa CD4 cell-tropic viruses, numerous HIV-specific syncytia were observed. For viruses that did not infect HeLa CD4 cells, no syncytia were observed.

variants allowed the generation of intertypic recombinant viruses to map the HIV genetic components which determine cell tropism. Surprisingly, the region of the viral genome associated with HBCE cell growth mapped to sequences within an 806-bp region (positions 5785 to 6591) of the NL4-3 genome which has not been previously correlated with cell tropism. Interestingly, HIV growth in HBCE cells was stringently determined by the presence of this NL4-3 sequence. This HIV genomic segment encodes five open reading frames which include the C1 region of env (amino acids 1 through 123), exon 1 of *tat* (tat-1), rev, vpu, and 19 amino acids at the C terminus of vpr.

Examination of the sequence differences between positions 5785 and 6591 in HIV strains which are permissive and nonpermissive for HBCE cell growth indicates that the majority of the significant amino acid changes occur in the C1 region of env (Fig. 4). Specifically, changes at positions 17, 31, and 87 demonstrated consistent differences between permissive and nonpermissive strains. The mutation at position 87 (Val or Gly to Glu) is of potential importance because this amino acid is adjacent to an N-linked glycosylation site at position 88 (28, 39). This glycosylation site is one of only 5 of the 24 potential N-glycosylation sites in gp120 that were found to influence infectivity (28). The nonconservative change of a valine or glycine in the permissive strains to a highly charged glutamic acid in the nonpermissive strains may influence N glycosylation. Since amino acid 87 is in a portion of the gp120 molecule that is exposed to the surface of the glycoprotein, either the glycosylation event or the mutation itself may play a role in viral attachment to HBCE cells.

Certain neuropathogenic retroviruses have been shown to exert neurotoxicity through infection of murine brain capillary endothelial cells (13, 30, 32). Similarly to our studies, a neuropathogenic variant of Friend murine leukemia virus (Friend MuLV), PVC-211 MuLV, was analyzed for viral sequences which mediate growth in murine brain capillary endothelial cells (32). Examination of chimeric viruses constructed from PVC-211 MuLV and the nonneuropathogenic parental Friend MuLV revealed that the major determinant for endothelial cell tropism was localized within an *Xba*I-*Bam*HI fragment in the *env* gene of PVC-211 MuLV. When this fragment was analyzed in more detail (31), substitution of only two amino acids was

FIG. 4. Amino acid sequence comparison of the *Sal*I-to-*Dra*III fragments of HBCE cell-tropic strains NL4-3, MN, and SF-2 and non-HBCE cell-tropic strains JR-CSF and SF-162. Sequences for the C1 region of env (amino acids 1 to 123), vpu, vpr, rev, and tat-1 are shown. The vpr sequence for SF162 is not available. Dots represent conserved amino acids. Position 87 in env C1 is boldfaced, and the N-linked glycosylation (NVT) site at positions 88 to 90 is underlined.

sufficient to confer endothelial cell tropism on the non-endothelial cell-tropic Friend MuLV. Thus, subtle changes within the envelope protein had a significant effect on the virus-host cell interaction. Similarly, discrete changes in the HIV envelope protein such as that in the C1 region of env may have a profound effect on the tropism of HIV for HBCE cells. Comparison of sequences from endothelial cell-tropic and -nontropic strains in the other open reading frames within the NL4-3 806-bp region which confers HBCE cell tropism indicates that only a small number of differential changes occur in the other genes. While vpr, vpu, tat, and rev may play a role in HBCE cell tropism, the mutations that occur in the tropic and nontropic strains are consistent with the C1 region of env being a major determinant of HIV infectivity.

The sequences responsible for determining T-cell and macrophage tropism are located primarily in the V3 loop of the env gene (5, 7, 10, 12, 17, 23, 41, 47, 54). Surprisingly, HBCE cell tropism did not cosegregate with tropism for either macrophages or immortalized cell lines, and determinants of HBCE cell tropism did not map to sequences within the variable regions of env, including the V3 loop. However, the V3 loop region of the HIV envelope glycoprotein gp120 was previously shown to be important for HIV infection of HBCE cells. In these studies virus infection was effectively neutralized with monoclonal antibodies to V3 loop determinants but not to the CD4 domain (35). Therefore, although the V3 loop may be important for interaction of the virus with the HBCE cellular receptor, this region does not appear to be a domain that selectively determines HBCE cell tropism.

While HBCE cell tropism did not cosegregate absolutely with tropism for macrophages or immortalized cell lines, three chimeric HIV clones (NCN-SN, NCN-SB, and NC-Dra) had dual tropism for both HBCE cells (Fig. 1) and macrophages (10). The increased neuropathogenicity of a neuropathogenic variant of Friend MuLV (NT40) has been correlated with an enhanced dual tropism for peritoneal macrophages, microglia, and brain endothelial cells (13). Similarly, macrophage-endothelial cell dually tropic HIV strains may possess an enhanced neuropathogenicity because of their expanded cellular tropism within the CNS. This possibility is further supported by the observation that, in vitro, contact between macrophages and HBCE cells was able to augment replication in macrophages (19).

Recently, we reported that endothelial cells cultured from bone marrow aspirates obtained from HIV-seropositive donors and patients with AIDS were significantly infected by HIV (38). Infection of bone marrow endothelial cells in vivo confirms the prediction that microvascular endothelial cells from certain tissues are a natural target for HIV. The observation that HIV infects bone marrow endothelial cells in HIV-seropositive patients without overt disease suggests that the virus establishes residence in these cells early in the disease process. Infection of bone marrow endothelium may predispose the generation of HIV strains which can infect brain endothelial cells.

HIV strains JR-FL and JR-CSF were originally isolated from the frontal lobe and cerebrospinal fluid of patient JR, who died with severe encephalopathy (27). The inability of brain-derived strains to infect HBCE cells implies either that such strains evolved within the brain parenchyma, losing their endothelial cell tropism after passage through the blood-brain barrier, or that non-endothelial cell-tropic strains in the peripheral blood entered the CNS via HIV-infected macrophages or lymphocytes. While brain-derived HIV isolates may represent a distinct subgroup, HBCE cells would not be exposed to such variants at the luminal surface, but, rather, would be exposed to HIV isolates present in the peripheral blood. Biological properties of peripheral blood isolates have been correlated with the progression of AIDS (8). Identification of HBCE cell-tropic HIV isolates in the peripheral blood may help predict when in the course of the disease process HIV is likely to enter the CNS.

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