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HIV-1 strain-associated variability in infection of primary neuroglia

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Qualitative differences among strains of Human Immunodeficiency Virus type 1 (HIV-1) may influence viral infectivity for cells of the central nervous system (CNS) and determine or at least significantly influence the neuropathogenesis of brain infection. In this study, we compared infectivity for these cells *in vitro* among several different laboratory-adapted HIV-1 strains differing in cellular tropism. These strains included three lymphotropic strains (SF2, NL4-3, and SG3.1), two macrophage-tropic strains (SF128A, SF162), and one brain-derived strain (YU2). In microglia, macrophage-tropic strain SF128A established productive infection while the lymphotropic strain SF2 did not. In infected astrocytes, all HIV-1 strains transiently produced variable and much lower levels of p24 antigen. Viral DNA *env* or *tat* gene sequences were amplified from infected astrocytes; the amplified signals varied among HIV-1 strains, but the strongest viral DNA signals were obtained from cells infected by the lymphotropic strains SF2 and SG3.1. Transfection of astrocytes with infectious HIV-1 proviral DNA clones confirmed the observation that HIV-1 strains differ in their ability to replicate in astrocytes. Transfection revealed post-entry blocks to replication by macrophage-tropic proviruses pSF128A and pSF162. However, cytomegalovirus (CMV) superinfection of transfected astrocytes enhanced p24 production by lymphotropic HIV-1 proviruses twofold and stimulated p24 production by the otherwise inactive macrophage-tropic proviruses. This study demonstrates the spectrum of HIV-1 strain-associated variation in infectivity for neuroglia, and suggests, in addition, that herpesviral factors or viral-induced cellular factors may stimulate HIV-1 infection in astrocytes and expand the neural cell tropism of certain HIV-1 strains.

Keywords: Human Immunodeficiency Virus; astrocytes; microglia; neuroglia; Herpesvirus infections; AIDS

Introduction

AIDS dementia complex (ADC) in HIV-1 infected individuals is characterized by astrocytosis, demyelination and neuronal loss (Sharer, 1992). However, very few cells in the brain are productively infected by HIV-1. Productive HIV-1 infection of the brain occurs predominantly in cells of the monocyte lineage, in particular microglia (Bagasara *et al*., 1996; Sharer, 1992; Wiley *et al*, 1986). These cells are the principal loci of HIV-1 structural gene expression, and they release both viral proteins and a variety of soluble inflammatory factors (Pulliam *et al*, 1991; Genis *et al*, 1992; Lipton and Gendelman, 1995; Fiala *et al*, 1996). Infection of these cells *in vitro* is characterized by HIV-1 virion production with or without cytopathic effect (Ioannidis *et al*, 1995). In contrast to productive infection in the microglia, restricted HIV-1 infection occurs predominantly in cells of neuroepithelial origin, in particular astrocytes (Tornatore *et al*, 1994a; Saito *et al*, 1994). During restricted infection, astrocytes express HIV-1 regulatory genes, in particular *nef*, with little or no structural gene expression (Tornatore *et al*, 1994a; Saito *et al*, 1994; Blumberg *et al*, 1994; Ranki *et al*, 1995). Restricted infection of astrocytes *in vitro* is characterized by predominant regulatory gene expression after an initial productive phase of structural gene expression and virion production (Tornatore *et al*, 1991, 1994b; Blumberg *et al*, 1994). The productive phase may be enhanced by concurrent herpesvirus infec-

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tion, which increases viral p24 antigen production (Ho *et al*, 1991). During the restricted phase of infection, structural gene expression may be reactivated by proinflammatory cytokines such as TNF-α or IL-1β (Tornatore *et al*, 1991, 1994b), and perhaps by opportunistic infections caused by coinfecting viruses.

HIV-1 isolates derived from the brain are usually tropic for monocytes/macrophages (Cheng-Mayer and Levy, 1990; Sharpless *et al*, 1992b), although isolates with a distinct microglia-tropic phenotype have been described (Strizki *et al*, 1996a, b). Moreover, the envelope protein sequences of HIV-1 brain-derived isolates are similar to those of macrophage-tropic HIV-1 strains (Power *et al*, 1995). Thus 'neurotropism' or 'neurovirulence' of HIV-1 isolates or strains has been defined in association with monocyte/macrophage-tropism and attendant infectivity for microglia (Power *et al*, 1995; Ioannidis *et al*, 1995; Sharpless *et al*, 1992a, b; Watkins *et al*, 1990; Cheng-Mayer and Levy, 1990; Koyanagi *et al*, 1987). However, restricted or infrequent productive HIV-1 infection of astrocytes has been reported in both pediatric and adult brains, and detectable astrocyte infection may be associated with moderate to severe HIV-associated dementing illness (Saito *et al*, 1994; Tornatore *et al*, 1994a; Ranki *et al*, 1995; Takahashi *et al*, 1996). Thus there is a need to further characterize HIV-1 infection of primary astrocytes and to determine whether there are any strain-associated variations in infectivity for CNS cells. Differences among HIV-1 strains may account for the very different types of infection that occur in microglia *versus* astroglia, and each may contribute to brain injury and clinical disease.

In this study, we found that human neuroglia, specifically low passage, nontransformed fetal astrocytes and adult microglia, could be infected *in vitro* by HIV-1, but that relative infectivity for these cells varied among several different laboratory-adapted HIV-1 strains. The replication in astrocytes of two macrophage-tropic strains, SF162 and SF128A, may be inhibited at the molecular level after viral entry and reverse transcription. Despite this variation in the infectivity for astrocytes by different HIV-1 strains, concurrent cytomegalovirus (CMV) infection of astrocytes ('superinfection') enhanced HIV-1 gene expression by all HIV-1 strains. This latter finding suggests that either a herpesviral factor or viral-induced cellular factors may play a role in stimulating HIV-1 infection in astrocytes and expanding the neural cell tropism of certain HIV-1 strains.

Results

HIV-1 strains with distinct cellular tropisms differ in infectivity for astrocytes and microglia

Two different HIV-1 strains, lymphotropic SF2 or macrophage-tropic SF128A, were first tested for their ability to infect astrocytes. No distinctive cytopathic effect (cpe) was observed in infected *versus* mock-infected astrocytes, but over a 48 h interval post-infection, transient soluble p24 antigen was detected in both SF2- and SF128A-infected culture supernatants (Figure 1A). Astrocyte cultures infected with strain SF2 produced three- to fivefold higher levels of soluble p24 antigen compared to astrocytes infected with SF128A even when up to four times as much SF128A inoculum was used (80 *versus* 20 ng p24 per 10^6 cells). In microglia,

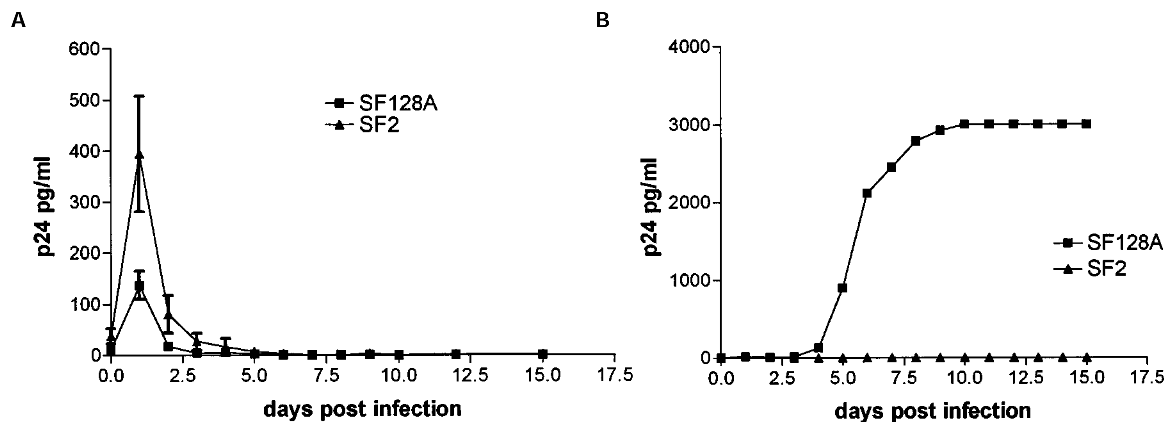

Figure 1 (**A**) Temporal course of soluble p24 antigen production after infection of human fetal astrocyte cultures with HIV-1 strains SF2 or SF128A. Data represent the mean ± s.e. from four independent experiments. Cell-free inoculum virus was used at 20–50 ng p24 SF2 antigen or 50–80 ng p24 SF128A antigen per 10^6 cells. Supernatants were collected every 24 h with complete change of culture medium. (**B**) Temporal course of soluble p24 antigen production after infection of human microglia cultures with HIV-1 strains SF2 or SF128A using cell-free inoculum virus at 20 ng p24 antigen per 10^6 cells. Supernatants were collected every 24 h with complete change of culture medium. Similar data were obtained from a second independent experiment using 50 ng p24 SF2 antigen per 10^6 cells.

however, macrophage-tropic strain SF128A established productive infection, with continued production of several thousand pg p24 antigen/ml from inoculum moi of 20 ng p24 per 10^6 cells (Figure 1B). No visible cpe was produced in SF128A-infected microglia over 15 days post infection. These data are consistent with published studies using highly enriched microglia cultures, suggesting productive HIV-1 infection occurs in microglia *in vitro*, with prolonged release of soluble p24 antigen (Lee *et al*, 1993; Ioannidis *et al*, 1995). In contrast, the lymphotropic HIV-1 SF2 strain did not establish transient or productive infection of microglia (Figure 1B).

The transient, comparatively low level of p24 antigen produced by infected astrocyte cultures could have derived from the release of bound HIV-1 inoculum, even though the infected astrocytes were extensively washed after initial virus adsorption. Accordingly, HIV-1 infection of astrocytes was confirmed by PCR amplification of viral *env* gene DNA sequences from infected astrocyte DNA extracted 3–5 days post infection. As shown in Figure 2 lanes 2 and 4, HIV-1 *env* sequences were detected in both SF2- and SF128A-infected cells. Even though the PCR analysis was not quantitative, the amplified DNA signal of SF128A, as detected by Southern blotting, was visibly weaker than that obtained with amplified viral DNA from SF2-infected cells. This was consistently observed in several independent experiments. Similar signals were detected with a pair of *tat* gene primers amplifying a sequence upstream of the *env* gene sequence and thereby representing a later event in viral reverse transcription (data not shown). Taken together with the p24 data, the PCR analysis suggested that the lymphotropic strain SF2 infects astrocytes much more efficiently than the macrophage-tropic strain SF128A.

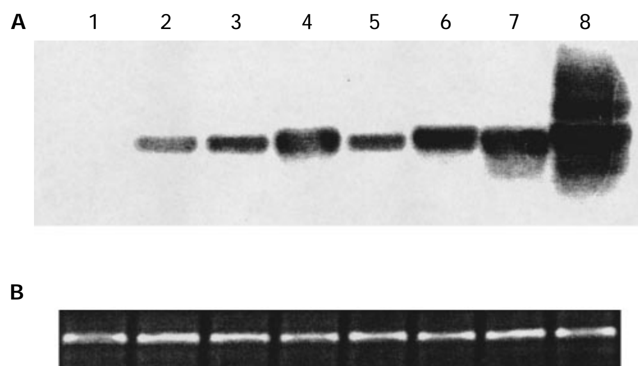

Figure 2 PCR amplification of HIV-1 *env* sequences in astrocytes infected by different HIV-1 strains (**A**). Astrocytes were infected with HIV-1, cellular DNA was extracted, and PCR amplification was performed as described in Materials and methods. Data are representative of four independent experiments. Astrocytes (10^6 cells) were infected with 7 ng p24 antigen of each viral strain. Lane 1 represents uninfected astrocytes; lane 2, SF128A; lane 3, SF162; lane 4, SF2; lane 5, NL4-3; lane 6, SG3.1; lane 7, YU2; lane 8, DNA extracted from the PBMC of an AIDS patient. (**B**) represents the signal from cellular actin DNA, amplified from each DNA extract in parallel to the viral gene DNA.

To further explore this apparent strain variation in HIV-1 infectivity for human neuroglia, additional HIV-1 strains were tested for their ability to infect human fetal astrocyte cultures. These included macrophage-tropic strain SF162, the brain-derived strain YU2, and two additional lymphotropic strains, NL4-3 and SG3.1. The additional HIV-1 strains transiently produced amounts of soluble p24 in infected culture supernatants ranging from about 25 to 65 pg/ml infected culture supernatant. Higher p24 production was not related to a viral strain's cellular tropism (data not shown). Infected astrocyte cultures were also compared by PCR amplification of viral DNA using the *env* primers as shown in Figure 2. Similar results were obtained using the *tat* primers. Astrocytes infected by the different strains of HIV-1 were found to contain detectable viral DNA sequences, suggesting that these HIV-1 strains enter the astrocyte, uncoat, and reverse transcribe RNA into viral DNA. However, the amplified viral DNA signals varied widely among different HIV-1 strains even though equivalent amounts of cellular DNA were used in the PCR reactions as normalized to the amplified cellular actin DNA (Figure 2B). Stronger viral DNA signals were obtained with cultures infected by the lymphotropic strains SF2 and SG3.1 or the 'dual' tropic strain YU2, while the weaker signals were obtained with cultures infected by macrophage-tropic strains SF128A and SF162 or the lymphotropic strain NL4-3. This additional comparative data, while not strictly quantitative and obtained with laboratory-adapted HIV-1 strains, further illustrated the HIV-1 strain-associated variability in infectivity for astrocytes.

Transfection of astrocytes with infectious HIV-1 proviral clones confirms strain differences in viral infectivity

In vitro studies of HIV-1 infection of human astrocytes are confounded by the inefficiency of virus uptake due to the lack of CD4 receptors on the cell surface (Gonzalez-Scarano *et al*, 1992). In order to study the replication of HIV-1 in astrocytes, transfection of proviral DNA directly into the cells has been demonstrated as a highly efficient means of introducing the infectious HIV-1 genome (Tornatore *et al*, 1991, 1994b). Transfection bypasses potentially inefficient virion entry into cells lacking the CD4 antigen receptor. Even though the transfection strategy is controversial (Spector *et al*, 1994), for it bypasses early events in viral replication (viral entry, uncoating, and reverse transcription) and potentially introduces a high viral DNA copy number per cell, we chose to use transfection to determine whether post entry events in the viral life cycle may be contributing to the strain-associated

variability in infectivity for astrocytes. Accordingly, infectious proviral DNA clones of the different strains of HIV-1 were transfected into human fetal astrocytes. No distinctive cpe was observed after transfection. Starting approximately 48 h post transfection (Figure 3), synthesis of soluble p24 antigen was detected in the cultures transfected with lymphotropic or pYU2 proviruses, but not in cultures transfected with macrophage-tropic proviruses, which produced only background levels of p24. To correct for any variation in transfection efficiencies among the provirus strains, transfection experiments were performed simultaneously with a β-galactosidase expression plasmid, and the p24 values were normalized according to the comparative β-galactosidase activities. The lymphotropic proviruses produced variable levels of p24 with maxima of 4500–6500 pg p24 per ml culture supernatant by 3–5 days post transfection. Then p24 production declined to baseline over a 1 week interval. The pYU2 provirus, which was cloned directly from uncultured human brain (Li *et al*, 1992) yielded a temporal p24 pattern in astrocytes which was intermediate between that of the macrophage-tropic proviruses (pSF128A, pSF162) and the lymphotropic proviruses (pSF2, pSG3.1, pNL4-3), with a similar time course and maximal p24 values of approximately 1300 pg/ml culture supernatant. These HIV-1 strain differences parallel those observed by Nath *et al* (1995) with infected astrocytes, again suggesting that infectivity for astrocytes may vary, at least approximately, according to the cellular tropism of the HIV-1 strains.

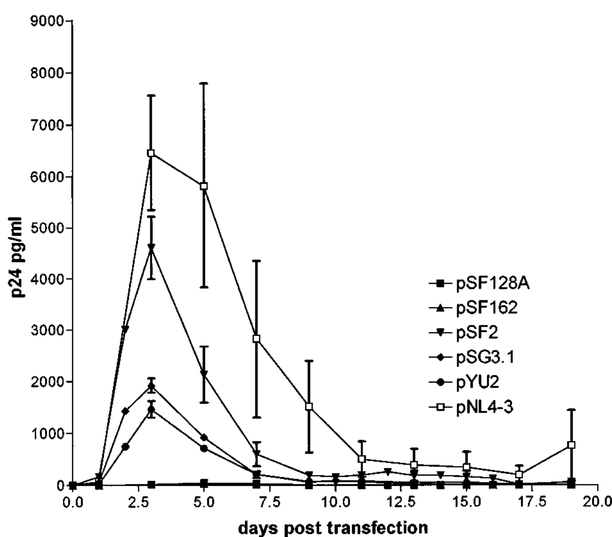

Figure 3 Temporal course of soluble p24 antigen production after proviral DNA transfection of human fetal astrocytes with different strains of HIV-1 proviruses. Cells were transfected with infectious molecular clones as described in Materials and methods. Culture supernatants were collected at transfection (time 0) and every 24 h with change of medium for quantitative p24 assay. Data represent the mean ± s.e. from three independent experiments.

To confirm that the proviral DNA of the different strains are non-defective, all the proviral DNA constructs were transfected into human peripheral blood mononuclear cells (PBMCs). Compared to astrocytes, transfected PBMCs produced 100-fold higher peak levels of p24 (Table 1), from approximately 50 ng/ml (pSG3.1) to 450 ng/ml (pSF162). Both macrophage-tropic strains, pSF128A and pSF162, generated levels of soluble p24 antigen that were comparable to those produced by the other strains. We had also previously observed that transfection of pSF128A into PBMCs or the rhabdomyosarcoma cell line RD4 generated similar levels of p24 as the T-cell tropic pSF2 (Liu *et al*, 1990). Thus the macrophage-tropic proviral DNA are functional, but apparently restricted in their infectivity according to cell type or cell lineage.

CMV superinfection enhances p24 production by both lymphotropic and macrophage-tropic strains of HIV-1 in transfected astrocytes

Herpesviruses represent an important group of potential agents in the neuropathogenesis of HIV-1, both as latent or indolent opportunistic viruses that increase AIDS morbidity and as viral co-factors capable of potentiating HIV-1 infection through molecular interactions in co-infected cells (Laurence, 1990; Kung and Wood, 1994). Herpesviruses, in particular CMV, have been implicated as important co-factors for HIV-1 infection. It is important to determine whether CMV infection of astrocytes affects strain-associated HIV-1 repliction in these cells since double-immunolabeling *in situ* techniques detecting viral nucleic acid indicate that CMV and HIV-1 infect the same cell in subcortical regions of the brain with a frequency of 12% of cells infected by CMV (Nelson *et al*, 1988). Primary or low passage human fetal astrocyte cultures will support productive infection *in vitro* by CMV (McCarthy *et al*, 1995; Ho *et al*, 1991). In cells transfected with proviral DNA from a lymphotropic HIV-1 strain (pHXB2), concurrent CMV infection has been reported to enhance p24 production

Table 1 Soluble p24 antigen production by HIV-1 provirus-transfected human peripheral blood mononuclear cells[a].

Provirus strain	*p24 antigen*
pSF128A	108
pSF162	450
pYU2	60
pSF2	130
pSG3.1	49
pNL4-3	128

[a]Values are expressed as ng p24 antigen per ml culture supernatant measured at the maximum of p24 production at 21 days post transfection.

approximately twofold (Ho *et al*, 1991). We compared the temporal course of soluble p24 antigen synthesis in lymphotrophic *versus* macrophage-tropic proviral DNA-transfected astrocytes which were concurrently 'superinfected' with CMV (Figure 4). In astrocytes transfected with lymphotropic pSF2, concurrent CMV infection stimulated more than a twofold increase in the peak p24 values during the initial productive phase of infection. In astrocytes transfected with macrophage-tropic pSF128A, concurrent CMV infection stimulated p24 antigen expression to levels comparable to those produced by astrocytes transfected with lymphotropic pSF2 in the absence of CMV infection. Thus concurrent CMV infection effectively overcame a post-entry block to pSF128A repliction in transfected astrocytes. This stimulatory effect of CMV superinfection was further confirmed in astrocyte cultures transfected by the other macrophage-tropic or lymphotropic HIV-1 proviruses (Table 2).

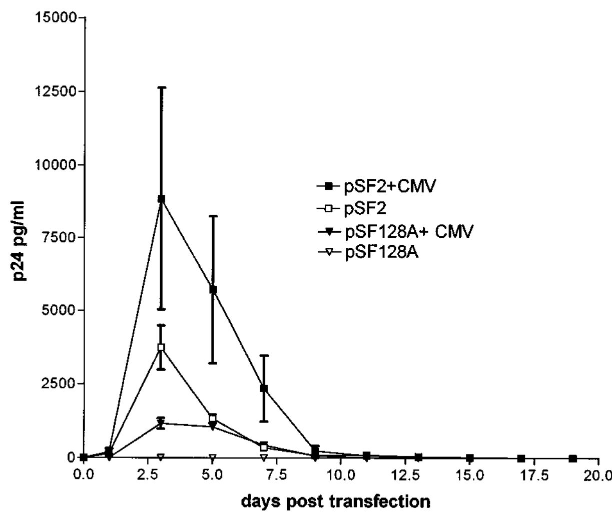

Figure 4 Effect of CMV infection on soluble p24 antigen production by pSF2- or pSF128A-transfected human fetal astrocytes. Subconfluent cells were mock- or pre-infected with CMV(AD169) at moi of 0.5 pfu/cell 4 h before transfection with the pSF2 or pSF128A proviruses as described in Materials and methods. Culture supernatants were collected at transfection (time 0) and every 24 h with change of medium for quantitative p24 assay. Data represent the mean ± s.e. of three independent experiments.

Table 2 Effect of CMV infection on peak soluble p24 antigen production by HIV-1 provirus-transfected human fetal astrocytes[a].

Provirus strain	*Provirus only*	*CMV infection*	*Fold stimulation*[b]
pSF128A	10 ± 5	1057 ± 159	106
pSF162	28 ± 21	187 ± 35	7
pYU2	1044 ± 449	4987 ± 852	5
pSF2	5049 ± 1012	11946 ± 5139	2
pSG3.1	3919 ± 929	6027 ± 2807	2
pNL4-3	5132 ± 765	10140 ± 3495	2

[a]Values are pg p24 antigen per ml culture supernatant measured at the maximum of p24 production within 3 – 5 days post transfection. Data represent the mean ± s.e. of three independent experiments. [b]Values are the quotient of peak p24 antigen with CMV infection divided by peak p24 with provirus only, rounded to the nearest integral value.

Discussion

This study demonstrates that among different strains of HIV-1, there is variable infectivity from primary neuroglia, specifically microglia and astrocytes. To a 'first approximation', infectivity for these cell types associates with distinct cellular tropisms of HIV-1 strains, i.e. monocyte/macrophage tropism and lymphotropism, respectively. Previous *in vitro* studies have indicated that productive HIV-1 infection in human microglia is readily established by monocyte/macrophage-tropic strains but not lymphotropic strains (Watkins *et al*, 1990; Sharpless *et al*, 1992a; Lee *et al*, 1993; Ioannidis *et al*, 1995; Strizki *et al*, 1996a, b). In our study, the macrophage-tropic strain, SF128A, originally derived from human spinal cord, established long-term productive *in vitro* infection in human adult microglia while the lymphotropic strain, SF2, did not establish productive infection at all. Monocyte/macrophage tropism is determined by sequences within the HIV-1 envelope protein (Liu *et al*, 1990; Chesebro *et al*, 1992). In so far as macrophage-tropism correlates with infectivity for microglia (Koyanagi *et al*, 1987; Watkins *et al*, 1990; Cheng-Mayer and Levy 1990; Sharpless *et al*, 1992a, b; Power *et al*, 1995; Ioannidis *et al*, 1995; Strizki *et al*, 1996b), infectivity for microglia may be determined by viral entry events mediated by the env protein. This may not be the case with HIV-1 strain infectivity for astrocytes. A recent study of HIV-1 isolates indicates that infectivity for astrocytes does not correlate with a particular sequence in the V3 loop of the viral envelope protein that is linked to macrophage tropism (Di Stefano *et al*, 1996a, b).

In contrast to the lack of infection of microglia, several lymphotropic strains have been observed to establish infection in astrocyte cultures, which yield relatively low quantities of virus (Cheng-Mayer *et al*, 1987; Cheng-Mayer and Levy, 1988; Nath *et al*, 1995). This is consistent with the concept of 'restricted' HIV-1 infection in astrocytes due to a relative predominance of regulatory *versus* structural gene transcription (Blumberg *et al*, 1994). In our human fetal astrocyte cultures, three lymphotropic HIV-1 strains directly infected astrocytes as evidenced by detection of virus-specific DNA sequences 3 – 5 days post infection. Consistent with recent reports (Nath *et al*, 1995; Di Stefano *et al*, 1996a), the amount of soluble p24 produced by the lymphotropic HIV-1-infected astrocyte cultures was

variable and very low, approximately tenfold lower than that produced by an equivalent number of HIV-1-infected microglia. Nevertheless, PCR assay consistently amplified viral *tat* or *env* gene sequences from cellular DNA whether or not p24 antigen production by these infected cells had ceased. Astrocyte cultures transfected with the three lymphotropic proviruses transiently produced soluble p24 antigen at levels comparable to those of human microglia cultures productively infected by macrophage-tropic HIV-1 strains. Among independent experiments, some variability in levels of p24 synthesis by different HIV-1 provirus strains was observed. This could be due to variation in transfection efficiency among proviral DNA constructs, although in this study transfection efficiency was normalized to co-transfected β-galactosidase reporter gene activity. In this study, a more likely source of variability in levels of p24 synthesis is the astrocyte cultures, which may reflect individual human variation. While the astrocyte cultures in a single experiment comparing multiple HIV-1 proviruses derived from an individual specimen, independent experiments used cells derived from distinct individual specimens. Cells from distinct specimens were never pooled. Specimen to specimen variability is a complicating factor in experiments using non-transformed, non-immortalized human neural cell cultures, since it is not possible to repeatedly obtain genetically identical CNS specimens.

By comparison with lymphotropic strains, the macrophage-tropic HIV-1 strains SF128A or SF162 could not efficiently or consistently infect the astrocytes when infectious proviral DNA was transfected into the cells. The two tested macrophage-tropic proviruses failed to produce measurable p24 antigen in transfected astrocyte culture supernatants. The relatively poor replication of these macrophage-tropic HIV-1 strains in astrocytes could be due to variable or unstable binding to the astrocyte surface at viral entry or to a block in one or more post-entry viral replication events. But transfection of macrophage-tropic proviral DNA into astrocytes resulted in no detectable infectious viruses, indicating that there is a block in viral gene expression after reverse transcription. The transfection data also offers compelling evidence that strain-associated HIV-1 infectivity for astrocytes is not solely determined by *env* sequences. Our CMV superinfection experiments further support the notion that certain macrophage-tropic HIV-1 strains cannot replicate independently after entry into the astrocyte. The presence of replicating CMV in astrocytes transfected with proviral DNA enhanced the replication of the lymphotropic HIV-1 strains and facilitated detectable replication of the macrophage-tropic strains pSF128A and pSF162. There are several possible mechanisms to explain this observation. Viral-induced cytokines such as TNF-α or IL-1β may be elaborated by herpesvirus-infected cells, and function to stimulate HIV-1 gene expression during restricted infection of astrocytes (Tornatore *et al*, 1991; Di Stefano *et al*, 1996a). CMV-encoded transcriptional factors or CMV-induced cellular transcriptional factors may be involved in the stimulation of the HIV-LTR in a variety of cell types (Ghazal and Nelson, 1993; Spector *et al*, 1994). There are reportedly HIV-1 strain-specific and cell-specific differences in transcription factor binding to regulatory sites on the LRT (Cannone-Hergaux *et al*, 1995); these may be influenced by concurrent CMV infection. Our ongoing studies suggest that in human fetal astrocytes HIV-LTR directed gene expression can be activated by CMV or human herpesvirus-6 via intracellular molecular interactions (McCarthy and Wood, manuscript in preparation). Some HIV-1 strains may require such transcriptional help to replicate in astrocytes. Our observations suggest that in the CNS, CMV superinfection can effectively expand the neural cell tropism and neurovirulence of HIV-1 strains by enabling or enhancing the infection of astrocytes, particularly by certain macrophage-tropic HIV-1 strains.

The viremia of primary HIV-1 infection may determine the initial seeding of virus in the CNS (Haynes *et al*, 1996). Viruses isolated during early HIV-1 infection tend to be more homogenous in their phenotypic and genotypic properties (Zhu *et al*, 1993). However, differential tropism for microglia *versus* astrocytes similar to that of laboratory-adapted HIV-1 strains has been observed with primary and low passage HIV-1 isolates. In a study of 27 virus isolates obtained during primary infection, Strizki *et al* (1996a, b) found that most isolates had overlapping monocyte/macrophage and microglia tropism. But they did identify a subset of isolates with distinct microglia tropism and an additional isolate that did not infect either monocyte-derived macrophages or microglia. Di Stefano *et al* (1996a, b) noted that approximately one third of 30 primary HIV-1 isolates could infect astrocytes *in vitro* as determined by PCR analysis. Thus there may be qualitative neurotropic differences between HIV-1 isolates that are apparent even early in the course of HIV-1 infection. Given the controversy surrounding the lack of correlation between the viral 'load' in the CNS and the clinical severity of such neurologic complications as dementia (Johnson *et al*, 1996), these qualitative differences that influence viral infectivity for the cells of the CNS may determine or at least significantly influence the neuropathogenesis of brain infection. Our studies suggest that there is a spectrum of qualitative neurotropic differences among HIV-1 strains defined by *both* viral entry and post-entry mechanisms. 'Neurotropism' and 'neurovirulence' may not be determined solely by

viral envelope sequence variations and viral entry-related events. Microglia harbor productive infection by monocyte/macrophage-tropic strains largely defined by envelope protein sequences. Astrocytes may harbor restricted HIV-1 infection by predominantly lymphocyte-tropic strains capable of temporally limited but re-activated viral transcription and replication. Superinfecting CMV can facilitate replication of all HIV-1 strains within this astrocyte 'reservoir,' thus expanding the neurotropism of the predominant microglia-tropic strains and thereby increasing the overall CNS viral load.

Materials and methods

HIV-1 strains and proviral clones
The lymphotropic HIV-1 strain SF2 was obtained from Dr J Levy (Luciw *et al*, 1984); the macrophage-tropic strain SF128A was derived as described previously (Liu *et al*, 1990). The macrophage-tropic proviral DNA clone pSF162 was obtained from Dr C Cheng-Mayer (York-Higgins *et al*, 1990). Two lymphotropic proviral DNA clones, pNL4-3 (Adachi *et al*, 1986) and pSG3.1 (Ghosh *et al*, 1993), and a third clone pYU2 derived from uncultured human brain tissue (Li *et al*, 1992), were obtained from the NIH AIDS Reference and Reagent Program (Rockville, MD, USA). The pYU2 clone replicates in monocytes/macrophages, primary T-lymphocytes, and the Molt4-clone 8 T-cell line; thus it has properties suggesting 'dual' cellular tropisms. To prepare stocks of infectious viruses for infection of astrocytes, proviral DNA clones were first transfected into CV-1 cells using cationic liposomes (Lipofectamine, GIBCO BRL, Gaithersburg, MD, USA) with 3 μg proviral DNA per 5×10^5 CV-1 cells. One week after transfection, 1 ml of CV-1 culture supernatant containing infectious HIV-1 viruses was then used to infect 2×10^6 normal human PBMCs stimulated with 5 μg/ml of phytohemagglutinin (PHA) for 24 h. Viruses produced by the PBMC infection were then quantitated in culture supernatants using a commercial p24 ELISA assay (Immunotech Inc, Westbrook, ME, USA).

Preparation of human neural cell cultures
Astrocyte cultures were prepared from human fetal CNS tissue of 46–83 days gestation. Fetal CNS tissue was obtained from the Human Embryology Laboratory, University of Washington (Seattle, WA, USA). Procedures for procurement and use of human fetal and non-fetal CNS tissue were approved and monitored by the University of Miami School of Medicine's Medical Sciences Subcommittee for the Protection of Human Subjects. The CNS tissue from each fetal specimen, consisting primarily of rhombencephalon and mesencephalon, was processed separately and independently, as were subsequent cell cultures; there was no pooling of CNS tissues from distinct fetal specimens. Highly enriched human fetal astrocyte cultures were prepared from mechanically dissociated CNS tissue as previously reported in detail (McCarthy *et al*, 1995). To avoid fibroblast overgrowth, cultures were adapted to serum-free B16 medium (Brewer and Cotman, 1989) supplemented with 5 ng/ml basic fibroblast growth factor (FGF2). For serial passage, cells were subcultured when confluent, approximately once per week. Infection or transfection studies were performed with cultures serially passaged between three and six times. Cultures were monitored by immunofluorescence assay (IFA) for expression of glial fibrillary acidic protein (GFAP), a marker for astrocytes, and fibronectin or prolyl-4-hydroxylase for fibroblast-type cells (McCarthy *et al*, 1995). Enriched human microglial cultures were prepared according to published methods (Whittemore *et al*, 1993) from non-fetal human lumbar spinal cord segments. Tissue was dissected free of blood vessels and meninges, dissociated with trypsin, and washed. Microglia were purified on Percoll gradients and recovered by differential adherence to plastic. They were maintained in B16 supplemented with 2.5% (v/v) fetal bovine serum (FBS) heat inactivated for 30 min at 56°C.

Viral infection and transfection of neural cells
To infect astrocytes or microglia directly with HIV-1 strains, 1×10^6 cells in T25 tissue culture flasks were first treated with 2 μg/ml polybrene, then incubated for 3 h at 37°C with PBMC-derived stock virus inoculum standardized as ng p24 antigen. After this adsorption of HIV-1 inocula, the infected cultures were washed with phsophate buffered saline (PBS) at least three times and then fed with fresh culture medium. Infected or mock-infected culture supernatants were collected every 24 h with change of culture medium for measurement of soluble p24 production by ELISA. Astrocytes were infected with cell-free preparations of human CMV strain AD169 as previously described (McCarthy *et al*, 1995), using 4×10^5 cells infected at a multiplicity of infection (moi) of 0.5 plaque forming units (pfu) per cell approximately 4 to 6 h prior to transfection with proviral DNA, or 4×10^5 cells infected at an moi of 1.0 pfu/cell 18 h post transfection. using either of these infection conditions, CMV antigen was present in greater than 80% of astrocytes in the culture by 48 h post-transfection as confirmed by IFA for viral structural antigens (McCarthy *et al*, 1995). CMV-infected monolayers remained intact and viable for 8–10 days. Fetal astrocyte cultures were transfected with molecularly cloned HIV-1 proviral DNA using cationic liposomes and 1 μg proviral DNA per $3-4\times10^5$ cells. Transfection of PBMCs was performed with 200 μg DEAE dextran per 2×10^7 cells. Variation in transfection efficiency among proviral DNA clones was monitored by co-

transfection of a reporter plasmid containing the CMV IE promoter (kindly provided by Dr Lung-Ji Chang, University of Alberta, Alberta, Canada) upstream of a β-galactosidase gene in the pT7T318U vector (Pharmacia Biotech, Piscataway, NJ, USA). β-galactosidase expression by transfected cells was determined by histochemical stain or enzyme assay. Transfected or mock-transfected culture supernatants were collected every 24 h with change of culture medium for measurement of soluble p24 production by ELISA. All reported values for p24 expression in proviral DNA-transfected cultures have been corrected for variations in transfection efficiency.

Amplification of HIV-1 viral DNA from infected astrocytes

To confirm HIV-1 infection of the astrocytes, the synthesis of HIV-1-specific DNA was verified by polymerase chain reaction (PCR) amplification of viral sequences from cellular DNA with the Taq (DNA) polymerase (Promega, Madison, WI, USA). HIV-infected astrocytes were harvested 1–8 days post infection for total cellular DNA using the IsoQuick Reagent (ORCA Research Inc, Bothell, WA, USA). PCR analyses used a pair of primers from the *tat* gene (5′-CATTTCAGAATTGGGTGTCGACATA-3′ and 5′-CCTAGTGTGTTGATAACGATAATAA-3′) or from the *env* gene (5′-GTATGAATTCAACTGCTGTTAAATGGCAGT-3′ and 5′-TATAGAATTCACTTCTCCAATTGTCCCTCAT-3′). The conditions for *env* and *tat* gene PCR were as follows: 3 μg DNA, 1 pmole/ml of each primer, 0.25 mM dNTP, 1.5 nM $MgCl_2$, 2.5 U of Taq polymerase in a 50 μl reaction volume. The reaction proceeded for 1 cycle at 94°C for 3 min, 50°C for 1.5 min, and 72°C for 2 min, followed by 35 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 2 min. As a positive control, the *tat* or *env* gene sequence was amplified from DNA extracted from H-9 cells or the productively infected PBMC of an AIDS patient. As an internal control to ensure that equal amounts of DNA were used for each amplification, PCR amplification of cellular actin using actin primers (5′-TTCTACAATGAGCTGCGTGT-3′ and 5′-GCCAGACAGCACTGTGTTGG-3′) was conducted in parallel to viral DNA amplification with all cellular DNA extracts. The PCR products and controls were analyzed on 1.2% agarose gels, and then hybridized to digoxigenin-labeled *env* or *tat* probes using the Genius kit (Boehringer Mannheim, Indianapolis, IN, USA). Amplified DNA signals were quantitated using the Biorad DOC 1000 fluorescence gel documentation system (Biorad, Hercules, CA, USA). The amount of each cellular DNA used for viral sequence amplification was normalized to the amplified actin signals.

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References

Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59:** 284–291.

Bagasara O, Lavi E, Bobroski L, Khalili K, Pestaner JP, Pomerantz RJ (1996). Cellular reservoirs of HIV-1 in the central nervous system of infected individuals: identification by the combination of *in situ* polymerase chain reaction and immunohisto-chemistry. *AIDS* **10:** 573–585.

Blumberg BM, Gelbard HA, Epstein LG (1994). HIV-1 infection of the developing nervous system: central role of astrocytes in pathogenesis. *Virus Res* **32:** 253–267.

Brewer GJ, Cotman CW (1989). Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. *Brain Res* **494:** 65–74.

Canonne-Hergaux F, Aunis D, Schaeffer E (1995). Interations of the transcription factor AP-1 with the long terminal repeat of different human immunodeficiency virus type 1 strains in Jurkat, glial, and neuronal cells. *J Virol* **69:** 6634–6642.

Cheng-Mayer C, Levy JA (1988). Distinct biological and serological properties of human immunodeficiency viruses from the brain. *An Neurol* **23:** S58–61.

Cheng-Mayer C, Levy JA (1990). Human immunodeficiency virus infection of the CNS: characterization of ‘neurotropic’ strains. *Curr Top Micro Immunol* **160:** 145–156.

Cheng-Mayer C, Rutka JT, Rosenblum ML, McHugh T, Stites DP, Levy JA (1987). Human immunodeficiency virus can productively infect cultured human glial cells. *Proc Natl Acad Sci USA* **84:** 3526–3530.

Chesebro B, Wehrly K, Nishio J, Perryman S (1992). Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell tropic isolates: definition of critical amino acids involved in cell tropism. *J Virol* **66:** 6547–6554.

Di Stefano M, Hinkula J, Fiore J, Scarlatti G, Major E, Chiodi F (1996a). Infection of human fetal astrocytes by primary HIV-1 isolates. *J Neuro Virol* **2:** 19.

Di Stefano M, Wilt S, Gray F, Dubois-Dalcq M, Chiodi F (1996b). HIV type 1 V3 sequences and the development of dementia during AIDS. *AIDS Res Hum Retroviruses* **12:** 471–476.

Fiala M, Rhodes RH, Shapshak P, Nagano I, Marinez-Maza O, Diagne A, Galdwin G, Graves M (1996). Regulation of HIV-1 infection in astrocytes: expression of Nef, TNF-α, and IL-6 is enhanced in co-culture of astrocytes with macrophages. *J Neuro Virol* **2:** 158–166.

Ghazal P, Nelson JA (1993). Interations between cytomegalovirus immediate-early proteins and the long terminal repeat of human immunodeficiency virus. *Rev Med Virol* **3:** 47–55.

Ghosh SK, Fultz PN, Keddie E, Saag MS, Sharp PM, Hahn BH, Shaw GM (1993). A molecular clone of HIV-1 tropic and cytopathic for human and chimpanzee lymphocytes. *Virology* **194:** 858–864.

Genis P, Jett M, Bernton EW, Boyle T, Gelbard HA, Dzenko K, Keane RW, Resnick L, Mizrachi Y, Volsky DJ, Epstein LG, Gendelman HE (1992). Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interations: implications for the neuropathogenesis of HIV disease. *J Exp Med* **176:** 1703–1718.

Gonzalez-Scarano F, Harouse JM, Kenny JJ, Kunsch C, Spitalnik SL, Wigdahl B (1992). Human immunodeficiency virus infection of neural cells. *Sem Virol* **3:** 225–234.

Haynes BF, Pantaleo G, Fauci AS (1996). Toward an understanding of the correlates of protective immunity to HIV infection. *Science* **271:** 324–328.

Ho WZ, Long L, Douglas SD (1991). Human cytomegalovirus infection and trans-activation of HIV-1 LTR in human brain-derived cells. *J Acq Immune Defic Synd* **4:** 1098–1106.

Ioannidis JPA, Reichlin S, Skolnik PR (1995). Long-term productive human immunodeficiency virus-1 infection in human infant microglia. *Am J Pathol* **147:** 1200–1206.

Johnson RT, Glass JD, McArthur JC, Chesebro BW (1996). Quantitation of human immunodeficiency virus in brains of demented and nondemented patients with acquired immunodeficiency syndrome. *Ann Neurol* **39:** 392–395.

Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen ISY (1987). Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science* **236:** 819–822.

Kung HJ, Wood C (1994). *Interactions between retroviruses and herpesviruses.* World Scientific Publishing Co. Singapore.

Laurence J (1990). Molecular interactions among Herpesviruses and human immunodeficiency viruses. *J Inf Dis* **162:** 338–346.

Lee SC, Hatch WC, Liu W, Kress Y, Lyman WD, Dickson DW (1993). Productive infection of human fetal microglia by HIV-1. *Am J Pathol* **143:** 1032–1039.

Li Y, Hui H, Burgess CJ, Price RW, Sharp PM, Hahn BH, Shaw GM (1992). Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. *J Virol* **66:** 6587–6600.

Lipton SA, Gendelman HE (1995). Dementia associated with the acquired immunodeficiency syndrome. *New Engl J Med* **332:** 934–940.

Liu ZQ, Wood C, Levy JA, Cheng-Mayer C (1990). The viral envelope gene is involved in macrophage tropism of a human immunodeficiency virus type 1 strain isolated from brain tissue. *J Virol* **64:** 6148–6153.

Luciw PA, Potter SJ, Steimer K, Dina D, Levy JA (1984). Molecular cloning of AIDS-associated retrovirus. *Nature (London)* **312:** 760–763.

McCarthy M, Wood C, Fedoseyeva L, Whittemore SR (1995). Media components influence viral gene expression assays in human fetal astrocyte cultures. *J Neuro Virol* **1:** 275–285.

Nath A, Hartloper V, Furer M, Fowke KR (1995). Infection of human fetal astrocytes with HIV-1: viral tropism and the role of cell to cell contact. *J Neuropath Exp Neurol* **54:** 320–330.

Nelson JA, Reynolds-Kohler C, Oldstone MBA, Wiley CA (1988). HIV and HCMV coinfect brain cells in patients with AIDS. *Virology* **165:** 286–290.

Power C, McArthur JC, Johnson RT, Griffin DE, Glass JD, Dewey R, Chesebro B (1995). Distinct HIV-1 *env* sequences are associated with neurotropism and neurovirulence. *Curr Top Microbiol Immunol* **202:** 89–104.

Pulliam L, Herndier BG, Tang NM, McGrath MS (1991). Human immunodeficiency virus-infected macrophages produce soluble factors that cause histological and neurochemical alterations in cultured human brains. *J Clin Invest* **87:** 503–512.

Ranki A, Nyberg M, Ovod V, Haltia M, Elovaara I, Raininko R, Haapasalo H, Krohn K (1995). abundant expression of HIV Nef and Rev proteins in brain astrocytes *in vivo* is associated with dementia. *AIDS* **9:** 1001–1008.

Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding K, Cvetkovich TA, Blumberg BM (1994). Overexpression of *nef* as a marker for restricted HIV-1 infection of astrocytes in post-mortem pediatric central nervous tissues. *Neurology* **44:** 474–480.

Sharer LR (1992). Pathology of HIV-1 infection of the central nervous system. A review. *J Neuropathol Exp Neurol* **51:** 3–11.

Sharpless ND, Gilbert D, Vandercam B, Zhou JM, Verdin E, Ronnett G, Friedman E, Dubois-Dalcq M (1992a). The restricted nature of HIV-1 tropism for cultured neural cells. *Virology* **191:** 813–825.

Sharpless ND, O'Brien WA, Verdin E, Kufta CV, Chen ISY, Dubois-Dalcq M (1992b). Human immuno-deficiency virus type 1 tropism for brain microglial cells is determined by a region of the *env* glycoprotein that also controls macrophage tropism. *J Virol* **66:** 2588–2593.

Spector DH, Koval V, Jault FM, Lathey J, Spector SA (1994). Positive and negative effects of human cytomegalovirus on HIV replication. In: *Interactions between retroviruses and herpesviruses.* Kung HJ, Wood C (eds). World Scientific Publishing Co: Singapore, pp 65–89.

Strizki JM, Albright AV, O'Connor M, Perrin L, Gonzalez-Scarano F (1996a). Differential tropism of acute HIV-1 isolates for monocyte derived macro-phages (MDM) and brain macrophages/microglia. *J Neuro Virol* **2:** 20.

Strizki JM, Albright AV, Sheng H, O'Connor M, Perrin L, Gonzalez-Scarano F (1996b). Infection of primary human microglia and monocyte-derived macrophages with human immunodeficiency virus type 1 isolates: evidence of differential tropism. *J Virol* **70:** 7654–7662.

Takahashi K, Wesselingh SL, Griffin De, McArthur JC, Johnson RT, Glass JD (1996). Localization of HIV-1 in human brain using polymerase chain reaction/in situ hybridization and immunocytochemistry. *Ann Neurol* **39:** 705–711.

Tornatore C, Nath A, Amemiya K, Major EO (1991). Persistent human immunodeficiency virus type 1 infection in human fetal glial cells reactivated by T-cell factor(s) or by the cytokines tumor necrosis factor alpha and interleukin-1 beta. *J Virol* **65:** 6094–6100.

Tornatore C, Chandra R, Berger JR, Major EO. (1994a). HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* **44:** 481–487.

Tornatore C, Meyers K, Atwood W, Conant K, Major EO (1994b). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68:** 93–102.

Watkins BA, Dorn HH, Kelly WB, Armstrong WC, Potts BJ, Michaels F, Kufta CV, Dubois-Dalcq M (1990). Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science* **249:** 549–553.

Whittemore SR, Sanon HR, Wood PM (1993). Concurrent isolation and characterization of oligodendrocytes, microglia, and astrocytes from adult human spinal cord. *Int J Devl Neuroscience* **11:** 755–764.

Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MBA (1986). Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci USA* **83:** 7089–7093.

York-Higgins D, Cheng-Mayer C, Bauer D, Levy JA, Dina D (1990). Human immunodeficiency virus type 1 cellular host range, replication, and cytopathicity are linked to the envelope region of the viral genome. *J Virol* **64:** 4016–4020.

Zhu T, Mo H, Wang N, Nam DS, Cay Y, Koup RA, Ho DD (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261:** 1179–1181.