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Analysis of human immunodeficiency virus type 1 gp160 sequences from a patient with HIV dementia: evidence for monocyte trafficking into brain

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Towards understanding the pathogenesis of HIV dementia, we molecularly cloned and sequenced human immundeficiency virus type 1 (HIV-1) gp160 genes from uncultured post-mortem tissues collected from a patient with HIV dementia. Sequences from bone marrow, lymph node, lung, and four regions of brain – the deep white matter, head of caudate, choroid plexus and meninges – were compared. Also included were gp160 sequences recovered from blood monocytes collected 5 months prior to death. Phylogenetic analyses showed that the sequences from deep white matter were more closely related to those from bone marrow, than to those from the other tissues, and moreover, were most closely related to sequences from the blood monocytes. These findings suggest trafficking of bone marrow-derived monocytes into the deep white matter during this late stage of infection. Another cluster included sequences from choroid plexus, meninges and lymph node, and interestingly, identical patterns of four or nine stop codons were shared among these tissues. These mutations appear to be the consequence of G→A hypermutation, and could reflect independent events, or the movement of virions or infected cells, from the choroid plexus into the cerebrospinal fluid and ultimately, into the lymph node. We propose that a critical step towards the development of HIV dementia is an increase in monocyte trafficking into the brain, and that this process is either initiated and/or accelerated during late-stage infection, which could explain why dementia occurs primarily during this time. *Journal of NeuroVirology* (2000) **6,** S70–S81.

Keywords: HIV; gp160; CNS; dementia; monocyte

Introduction

Typically, HIV-associated neurological disease, particularly dementia, does not present prior to immunodeficiency and if it does, tends to progress slowly (McArthur *et al*, 1989). The reasons for this still remain elusive. Studies of cerebrospinal fluid and brain tissue indicate that both HIV and Simian Immunodeficiency Virus (SIV) infect the central nervous system (CNS) early (Ho *et al*, 1985; Davis *et al*, 1992; Hurtrel *et al*, 1991). Although these viruses appear to enter the brain during acute infection or shortly thereafter, it is unclear whether this entry leads to the establishment of significant viral persistence. Indeed, no HIV proteins and no or only very low levels of HIV DNA have been found in the brains of asymptomatic individuals (Bell *et al*, 1993; Donaldson *et al*, 1994; Kibayashi *et al*, 1996). Interestingly, in a serial sacrifice study of macaques inoculated with the pathogenic SIV-mac251, Hurtrel and colleagues observed that intravenous, but not intracerebral (IC) inoculation, led to detectable brain infection, as well as florid neuropathology, at 2 and 3 months post inoculation (PI) (Hurtrel *et al*, 1991). Among the IC-inoculated animals, SIV antigen was detected in the brain at day 7, but not at 1 or 3 months PI, although all of these animals had persistent systemic infection. Similarly, Baskin and colleagues found that intrathecal inoculation did not lead to an increase in SIV-related neurological disease (Baskin *et al*, 1992). These observations suggest that maintenance of SIV infection within the brain, at least within the early months following inoculation, is dependent upon reseeding. An analogous situation is likely in HIV infection. This reseeding could be continual or sporadic and/or associated primarily with the onset

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of AIDS. Presumably, either infected cells or cell-free virions could participate.

A number of studies have compared sequences from the hypervariable region 3 (V3) of HIV gp160 recovered from brain tissues, to V3 sequences from blood or other tissues (Epstein *et al*, 1991; Korber *et al*, 1994; Power *et al*, 1994; Reddy *et al*, 1996; Di Stefano *et al*, 1996; Chang *et al*, 1998). Several of these have concluded that the HIV quasispecies in brain constitute a distinct group, and that this reflects independent evolution of the virus here. In addition, a brain signature sequence within V3 has been suggested (Korber *et al*, 1994), and particular V3 determinants associated with HIV dementia have been proposed (Power *et al*, 1994), although the latter has been questioned (Di Stefano *et al*, 1996). Other investigators, however, have found HIV variants in brain or cerebrospinal fluid (CSF) to be similar to those in lymphoid tissue (Keys *et al*, 1993; Reddy *et al*, 1996). Thus, the existence of specific neurotropic or neuropathogenic strains of HIV remains controversial, although there is general agreement that the HIV strains in brain are macrophage tropic.

The V3 region comprises a very small portion of the gp160 envelope gene, so although determinants important for cell tropism, neutralization and other biological behaviors map to this region, it may be limited in its ability to provide an accurate picture of the evolutionary relationships between HIV quasispecies in brain and those in other tissues. Such information can help us to determine at what stage(s) of HIV infection, and by what route, the brain becomes infected, and perhaps also provide information regarding the possible selection pressures operational. In this study, we analyzed HIV gp160 sequences from the brain and other tissues taken from a patient with HIV dementia. We show that gp160 sequences from the deep white matter (DWM) are more closely related to those in bone marrow (BM), than to those in the choroid plexus (CP), meninges (men), lymph node (LN), lung, or the head of the caudate (HC). Moreover, gp160 sequences recovered from blood monocytes collected 5 months prior to death cluster with the DWM/BM group and are, in fact, those with highest homology to the DWM sequences. These findings suggest the trafficking of infected monocytes from bone marrow to the brain, in late-stage HIV infection.

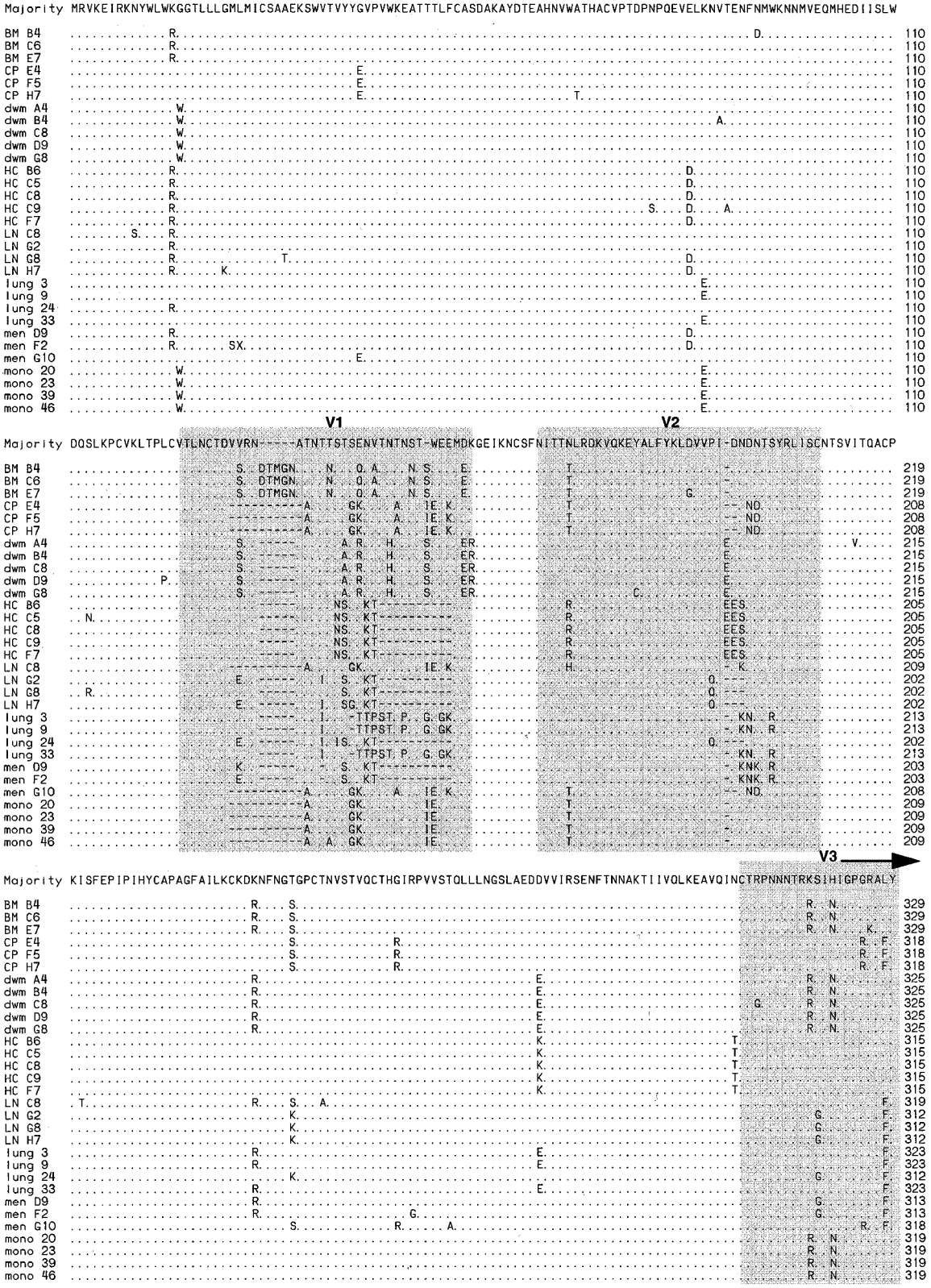
Results

Comparison of gp160 genes recovered from different tissues

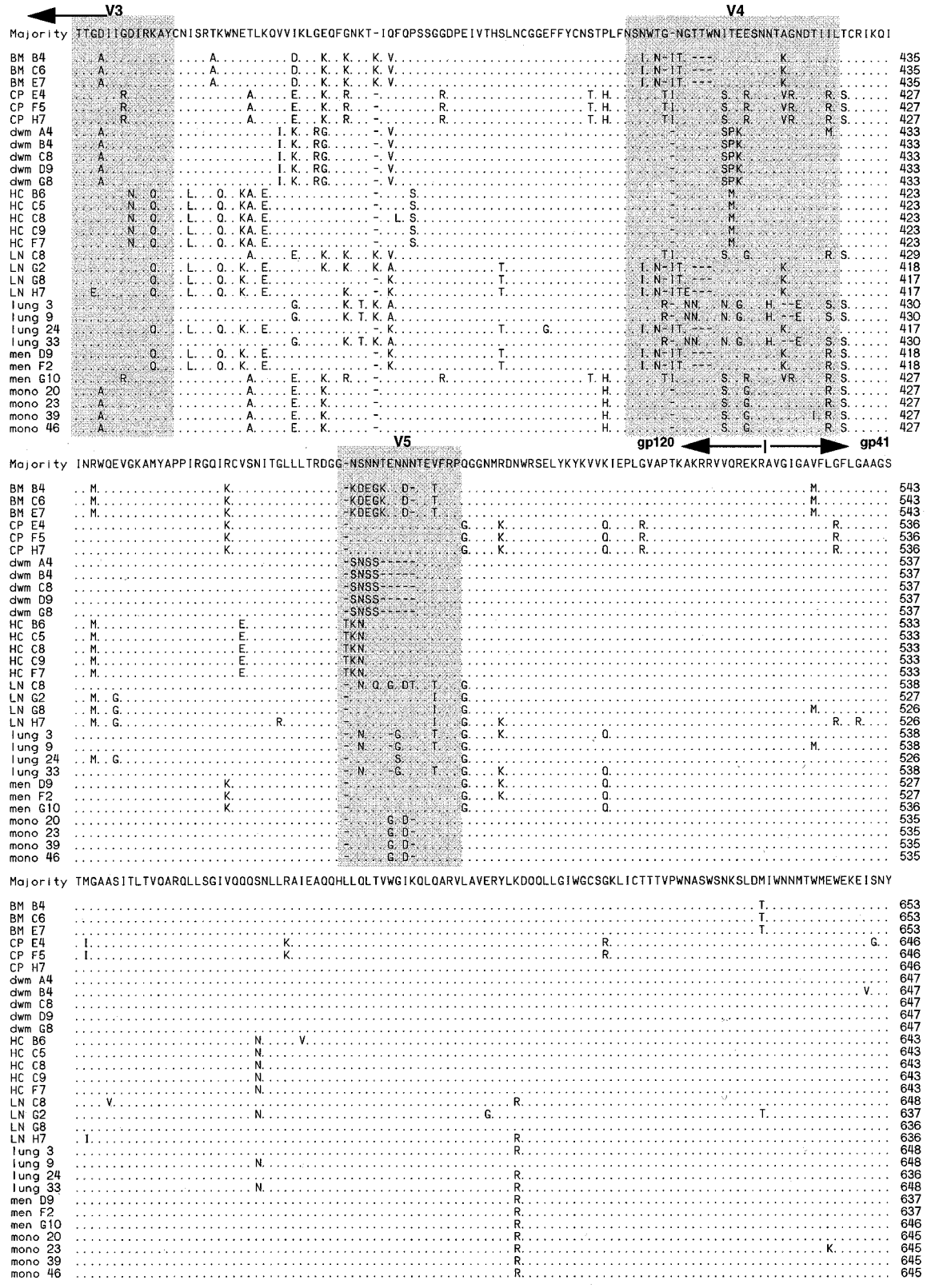
To investigate the breadth and nature of HIV-1 quasispecies present, we amplified gp160 genes from several tissues using the polymerase chain reaction (PCR), and molecularly cloned and sequenced these. Subsequently, gp160 genes were amplified from monocytes purified from a peripheral blood specimen taken from this patient 5 months prior to death. An alignment of the predicted amino acid sequences from 31 clones is shown in Figure 1. Included are three clones each from BM, CP and meninges, four clones each from LN, lung and the blood monocytes, and five clones each from DWM and HC. All clones from BM, DWM, lung and the blood monocytes have intact reading frames. Clone B6 from HC has one stop codon and clone F7 from HC has a one nucleotide deletion leading to a frameshift. The complete reading frames from the other three HC clones are intact. One of the three LN clones (H7) has an interrupted reading frame, consisting of 13 stop codons, and all three clones recovered from both the CP and the meninges lack complete open reading frames. CP clones E4 and F5 both have 11 stop codons, and CP clone H7 has 5 stop codons. Meninges clone D9 has one stop codon, clone G10 has four stop codons, and clone F2 has a one nucleotide deletion resulting in a frame shift. Almost all of these stop codons are TAG. At this position, the other clones have TGG codons, suggesting that the stops are the result of G→A hypermutation. Surprisingly, identical patterns of stop codons are present among the three CP clones, meningeal clone G10 and LN clone H7. These codons are located at amino acid positions 33, 67, 110 and 501 (numbers refer to consensus sequence positions). Choroid plexus clones E4 and F5 and LN clone H7 share additional stop codons at positions 593, 618, 645 and 653, and CP clones E4 and F5, LN clone H7 and meninges clone D9 share a stop at position 692. These patterns are diagrammed in Figure 2.

As expected, the majority of differences present within the gp120 region are located within the five hypervariable regions. Length polymorphisms are present in all of these except hypervariable region 3 (V3). Of note, all clones from the BM, DWM and blood monocytes are identical within the V3, except for one position in BM clone E7. This motif is not present in clones from any other tissue. More variability is seen within V4 than V3, and some patterns of these differences are identical among clones from different tissues. For example, a set of differences which includes six positions, as well as a deletion of three contiguous amino acids, is present in all three BM clones, in LN clones G2, G8 and H7, lung clone 24 and meningeal clones D9 and F2. A number of differences are also within the region between V3 and V4, including one length polymorphorism. For the most part, within this region, the BM, CP, DWM and monocyte clones group together and the HC, LN and meninges clones group together. Three of the four lung clones form one other group. Lymph node clone C8 diverges from the other LN clones, and the pattern of differences is more similar to that within the BM and CP groups, rather than that characteristic of the other LN clones. Within V5, all clones from DWM

exhibit a deletion of five contiguous amino acids, relative to the consensus. This is not present in any clones from other tissues. No pattern of V5 changes is common to clones from different tissues. Similarly, no distinct pattern of differences is apparent within V2. A detailed description of V1 variability



is presented below. Some differences are present at positions within the gp41 region. No characteristic patterns are apparent, although many (relative) changes are found in most or all clones from a given tissue, and some are present in clones from more than one tissue.



A summary of the genetic divergence among all clones is provided in Table 1. The degree of divergence at the nucleotide level is shown for both the entire gp160 gene and for the gp120 portion, independently. Regarding intra-tissue variation, only minimal divergence is present within the groups from BM, DWM, HC and the monocytes. This is in contrast to LN, where a significantly higher percentage of divergence is present (0.1–0.3% for the BM/DWM/HC/monocyte group *versus* 3.5% for LN). Variation within the lung group and the group from meninges is also considerably higher than for the BM/DWM/HC/monocyte group. Of particular note, the sequences from DWM are more closely related to the blood monocyte sequences than to sequences from other tissues, including those from other regions of brain. Moreover, the percentage of divergence between the DWM and monocyte groups is considerably less than that between any other two groups, except for the CP *versus* meninges comparison. Even then, for the entire gp160 gene, the DWM/monocyte divergence is 2.2% compared to 2.7% for the CP/meninges comparison. Other inter-tissue variations range from 3.0 to 5.4%, when both gp120 and gp160 sequences are taken in account.

Phylogenetic tree analysis

To better understand the evolutionary relationships among the clones, we performed phylogenetic analyses. The tree obtained, based on gp160 nucleotide sequences, is shown in Figure 3. As can be seen, the sequences from DWM and the monocytes cluster together, indicating greater

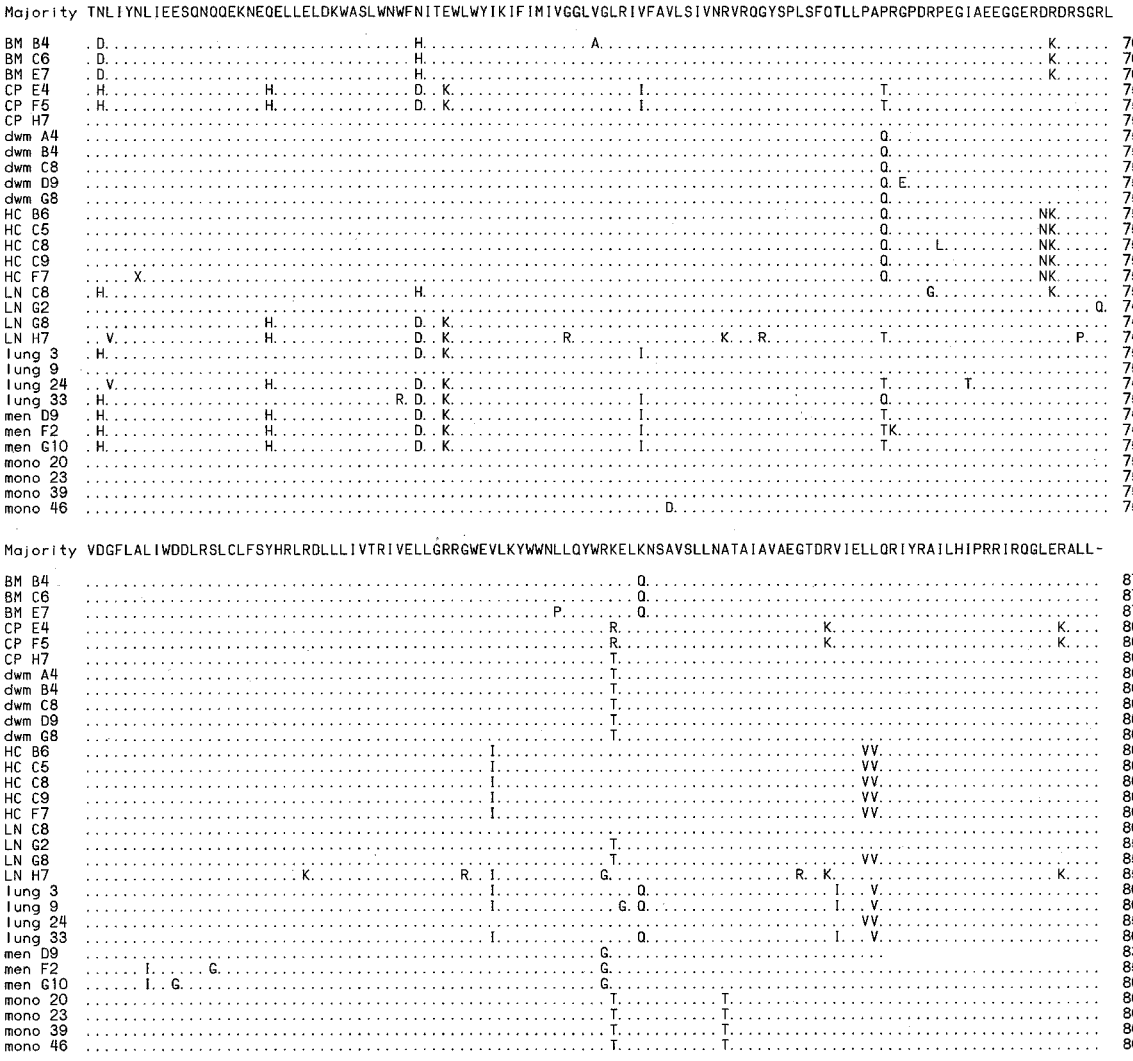


Figure 1 Alignment of predicted amino acid sequences from HIV-1 gp 160 clones recovered from tissues and blood monocytes from a patient with HIV dementia. BM, bone marrow; CP, choroid plexus; dwm, deep white matter; HC, head of the caudate; LN, lymph node; men, meninges; mono, blood monocytes. The hypervariable regions are shaded and identified as V1–V5. Sequences were first aligned using the Clustal program, then visually inspected and manually refined. Sequence lengths are shown to the right of the alignment.

homology between the HIV-1 species in these groups, as well as a more recent evolutionary divergence between them, relative to the species in other tissues. The tree also shows that the BM sequences cluster with the DWM/monocyte group, although the BM species clearly diverged at an earlier time. A second cluster contains the CP sequences, as well as one from the LN and another from the meninges. The other LN sequences cluster with one sequence from lung, two from the meninges, and more distantly, all of the HC sequences. The remaining three lung sequences cluster together and form a separate group, more distantly related to all others.

The V1 hypervariable region and length polymorphism

Among the gp160 sequences recovered from this patient, the most extensive and variable differences in length are located within the V1 region. As can be seen in Figure 4, some common patterns are apparent (see also Figure 1). Based on amino acid identity and the length and location of (arbitrarily

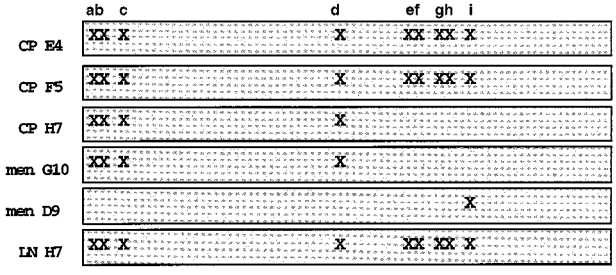


Figure 2 Schematic diagram of stop codons present in gp160 clones from choroid plexus, meninges and lymph node. CP, choroid plexus; men, meninges; LN, lymph node. Stop codons are indicated by X's. The positions are numbered as shown in Figure 1 and identified in this diagram by letters. These are: positions 33 (a), 67 (b), 110 (c), 501 (d), 593 (e), 618 (f), 645 (g), 653 (h) and 692 (i).

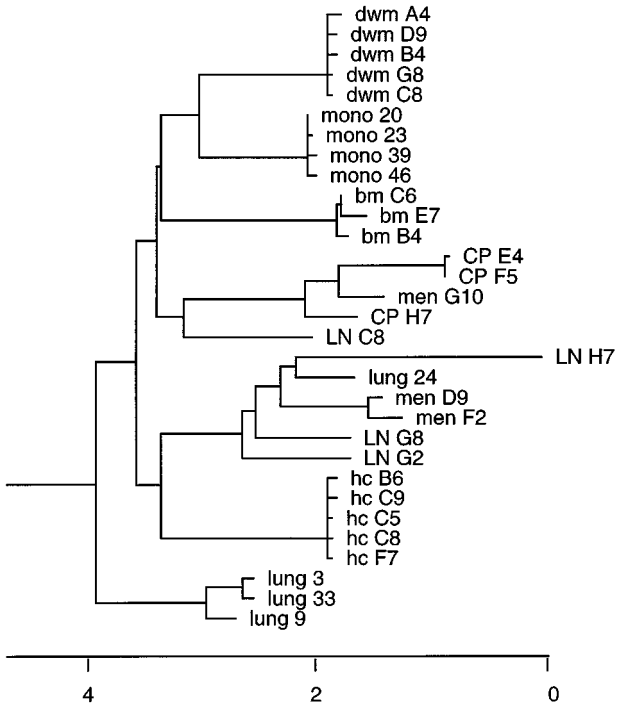


Figure 3 Phylogenetic tree of gp160 nucleotide sequences from patient 546. Sequences were aligned using the Clustal program, then visually inspected and manually refined prior to construction of the tree. The neighbor-joining method was used for the analysis and the tree was rooted upon the gp160 sequence from HIV-1_{ELI}. Gaps were not scored. The length of each pair of horizontal branches represents the distance between sequence pairs and can be read as a per cent difference using the scale bar. Vertical lengths are for clarity only.

Table 1 Per cent nucleotide divergence among HIV-1 envelope genes from tissues of Patient 546

Tissue		*Bone marrow*	*Choroid plexus*	*White matter*	*Caudate*	*Lymph node*	*Lung*	*Meninges*	*Monocytes*
BM[a]	gp160	0.2 (0.1–0.3)[b]							
	gp120	0.3 (0.1–0.4)							
CP	gp160	4.2 (3.7–4.6)	1.0 (0–1.5)						
	gp120	5.0 (4.9–5.3)	0.1 (0–0.1)						
WM	gp160	3.2 (3.1–3.4)	4.0 (3.2–4.4)	0.1 (0.1–0.2)					
	gp120	4.0 (3.9–4.3)	4.8 (4.8–4.9)	0.1 (0.1–0.2)					
HC	gp160	3.9 (3.8–4.1)	4.6 (3.9–4.9)	3.3 (3.3–3.4)	0.1 (0.1–0.2)				
	gp120	5.4 (5.3–5.7)	5.3 (5.3–5.5)	4.5 (4.4–4.5)	0.1 (0–0.2)				
LN	gp160	3.8 (2.9–5.3)	4.2 (2.8–5.0)	4.0 (3.0–5.7)	3.6 (3.0–4.8)	3.4 (1.7–5.0)			
	gp120	4.8 (4.1–5.8)	5.0 (3.4–5.7)	5.2 (4.0–6.2)	4.5 (3.8–4.9)	3.5 (1.7–5.6)			
Lung	gp160	3.6 (3.3–3.8)	3.9 (3.5–4.3)	3.5 (3.3–3.9)	3.7 (2.9–4.2)	3.5 (1.5–5.3)	2.1 (0.2–3.7)		
	gp120	4.7 (4.4–5.0)	4.5 (4.2–5.1)	4.5 (4.2–5.0)	5.0 (3.5–5.7)	4.1 (1.2–5.6)	2.5 (0–4.9)		
Men	gp160	4.0 (3.7–4.3)	2.7 (1.3–4.1)	4.0 (3.8–4.3)	4.0 (3.8–4.3)	3.5 (2.3–4.8)	3.3 (1.9–3.9)	2.1 (0.4–2.9)	
	gp120	5.1 (4.8–5.5)	2.5 (0.3–4.8)	4.9 (4.6–5.3)	4.8 (4.3–5.3)	4.0 (2.3–5.5)	4.1 (1.9–4.9)	3.2 (0.3–4.8)	
Mon	gp160	3.2 (3.0–3.3)	3.1 (2.4–3.5)	2.2 (2.1–2.3)	3.4 (3.4–3.5)	3.8 (2.4–5.5)	3.4 (3.2–3.7)	3.5 (2.7–4.2)	0.1 (0–0.2)
	gp120	3.7 (3.6–4.0)	3.0 (2.9–3.2)	2.8 (2.8–3.0)	4.2 (4.1–4.4)	4.7 (3.0–5.9)	4.1 (3.7–4.7)	3.9 (2.8–5.2)	0.1 (0–0.1)

[a]BM, bone marrow; CP, choroid plexus; WM, deep white matter; HC, head of caudate; LN, lymph node; men, meninges; mono, blood monocytes collected 5 months prior to death. [b]The values shown are for the mean and range (in parentheses) among clones for the groups specified. Only single nucleotide differences were scored; gaps were not scored. The sequence from meninges clone D9 has a small deletion (69 nucleotides) at the 3′ end, and was not included in the inter-tissue analyses, but was included in the intra-meninges calculations.

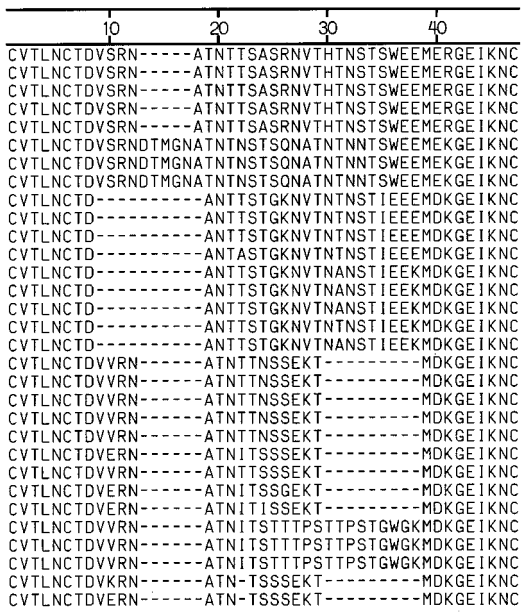


Figure 4 Alignment of the predicted amino acid sequences from the V1 loop of HIV-1 gp160 clones recovered from patient 546. Abbreviations are: deep white matter, dwm; BM, bone marrow; mono, peripheral blood monocytes collected 5 months earlier; CP, choroid plexus; LN, lymph node; men, meninges; HC, head of caudate. Sequences were aligned as described in the Figure 1 legend.

defined) deletions, the sequences can be grouped into four sets. (For the following descriptions of differences in length, the BM sequences, which are the longest, are used as the point of reference). One set includes the DWM and BM sequences and is characterized by the following motif: a serine residue at position 10, an alanine-threonine pair at positions 18 and 19, a serine at position 35 and a glutamic acid at position 40. A second set includes the monocyte and CP sequences, as well as the LN C8 and meninges G10 sequences. This set is characterized by a deletion of ten contiguous amino acids, a glycine-lysine pair at positions 25–26, and an isoleucine-glutamic acid pair at positions 35–36. A third group includes the HC series, LN clones G2, G8 and H7, meninges clones D9 and F2 and lung clone 24. This group can be distinguished by the following pattern: deletions of six and 10 contiguous amino acids, a threonine-asparagine pair at positions 20–21, a threonine at position 23, and a glutamic acid-lysine pair at positions 27–28. The other three lung clones (3, 9 and 33) form the fourth group. Unique to this group is the sequence TTTPSTTPSTGWGK spanning positions 25–38, and including a tandem repeat.

Discussion

We found HIV species in the DWM region of post-mortem brain to be significantly more closely related, genetically, to those recovered from blood monocytes collected 5 months earlier, than to those present concomitantly in the caudate, choroid plexus, meninges, bone marrow, lung or a subclavicular lymph node. This was true both for the full-length gp 160 gene, as well as the gp120 portion evaluated independently. The viral species from DWM were also more closely related to those in BM than those in the other tissues, the DWM, monocyte and BM sequences clustering together as a group. One interpretation of these findings is that marrow-derived monocytes within the circulation entered the parenchyma of the DWM and were either retained there, most likely as perivascular macrophages, and/or transmitted the infection to other neighboring cells. Several relevant facts support this possibility: (1) although the number of circulating monocytes infected with HIV is generally low (Shen *et al*, 1994), monocyte production and trafficking, both into and out of the circulation, is a very dynamic process, even under normal conditions. Experimental evidence indicates that monocytes exiting from bone marrow spend only 5–10 h in blood before migrating into the tissues (Meuret and Hoffman 1973; Meuret *et al*, 1974), and the blood monocyte turnover rate has been calculated to be 7×10^6 cells/kg/h (Meuret and Hoffman 1973); (2) while it is thought that resident parenchymal microglia represent a fixed population, evidence in humans, as well as rodents, indicates that perivascular macrophages, which are situated on the parenchymal side of the blood-brain barrier (BBB), are continually repopulated from the bone marrow (Hickey and Kimura 1988; Unger *et al*, 1993). This occurs at low levels under normal conditions, and is accelerated during CNS inflammation (Lassmann *et al*, 1993). The frequent perivascular location of HIV-positive multinucleated giant cells (MNGC) bearing macrophage markers indicates that the perivascular macrophage is a host for HIV infection, and recently, it has been suggested that this cell is the primary target for SIV infection in the CNS (Williams *et al*, 1999) and (3) the ratio of monocytes to CD4+ T-lymphocytes in the blood specimen studied was 1.9 – that is, almost twice as many monocytes were present as CD4 T-cells. Moreover, 32% of these circulating monocytes were CD16+, indicating that they were activated, which could conceivably enhance migration across the endothelium. HIV dementia has been shown to correlate with the presence of increased numbers of CD16+ monocytes (Pulliam *et al.*, 1997; Gartner *et al.*, 1999). Cloning and sequencing of the HIV species present within the T-lymphocyte fraction of the ante-mortem blood specimen are currently underway. The three gp120 T-cell clones sequenced thus far do not cluster with the BM/DWM/monocyte group. Moreover, the degree of divergence between the BM, DWM and monocyte gp120 sequences and those from the T-cells, ranges from between 5.2–6.6% for two of the

T-cell clones, and 12.4–13.1% for the other. Also, given that we must account for genetic similarity between the blood and bone marrow species, coupled with the facts that (1) the number of CD4+ T-lymphocytes in bone marrow is low, and (2) these cells do not generally traffic out of the marrow via the blood, it seems unlikely that the sequences we are ascribing to the monocyte population are actually derived from contaminating T-cells.

Based on studies of HIV p17 and the flanking (not hypervariable) regions of V1/V2 in three AIDS patients, Hughes and colleagues found that the ages of the viral populations within the lymphoid tissues ranged from 2.65–5.6 years as compared to 4.1–6.2 years in brain tissues (Hughes *et al*, 1997). They suggested that infection of the brain in these patients may have occurred early, preceding the onset of AIDS, or alternatively, that viral diversity within brain could be the result of multiply entries from sources outside the CNS. If multiple entry occurs, the actual duration of infection in brain may be considerably shorter than that indicated by estimates of population diversity. Our findings suggest that bone marrow is a source of HIV infection of brain. Little is known about the frequency and extent of infection in bone marrow, the kinetics of virus replication in this tissue, and the consequent breadth of genetic variation. Conceivably, ongoing replication in marrow, coupled with the regular exit of monocytes harboring these genomes, the entry of some of these into the brain, along with, in some cases, further transmission and replication of these viral species within brain, could be sufficient to account for a significant degree of intrabrain genetic diversity. Interestingly, the brain of a neurologically asymptomatic patient, dying following a fulminant course of aplastic anemia, contained many perivascular MNGC; the bone marrow showed severe cell depletion with approximately one in every 30–50 cells HIV positive (Vinters *et al*, 1988). Thus, we believe that a multiple entry process of HIV into the brain, at least during late-stage disease, is likely. Reactivation of latent virus seems less plausible, for several reasons: (1) the number of latently-infected cells would appear to be miniscule, since the levels of HIV DNA in the brains of asymptomatic individuals are exceedingly low (Bell *et al*, 1993; Donaldson *et al*, 1994). Some of the macrophages and microglial cells, albeit long-lived, will surely die; so, unless some replication occurs, the infected cells will gradually be replaced by uninfected ones. However, HIV expression is almost never detected in asymptomatic brains (Kibayashi *et al*, 1996). Efficient spread of HIV within the brain parenchyma may also be compromised by the fact that the primary target cells are frequently at some distance from one another. These observations question whether the reservoir of latently-infected cells in brain is sufficient to serve, upon activation, as the seed for the levels of viral DNA detectable in late-stage disease. It should be noted, however, that unlike the case we present here, in the three brains studied by Hughes and colleagues, there was no evidence of perivascular or leptomeningeal inflammatory infiltrates (Hughes *et al*, 1997). This may indicate differences in timing and mode of entry of the virus into the brain.

It is unclear as to why the HC clones do not also group with the DWM/monocyte/BM group. Perhaps fewer of the circulating monocytes entered the HC, or the level of replication there was greater, thereby leading to greater diversity. Greater compromise of the BBB may have been present within the DWM (Power *et al*, 1993), permitting influx of greater numbers of monocytes. Interestingly, in a study of another patient, we recovered one gp160 clone from DWM that was very closely related to three clones from BM, the level of divergence being only 0.3–0.4% at the nucleotide level. No clones highly related to BM were recovered from the parietal lobe of this brain (data not shown). Similar comparisons of sequences from other patients are required, but our observations may have significance, especially given the fact that HIV dementia is primarily a white matter disease.

Based on comparisons of the V3 region of gp160, a tissue-specific distribution of HIV species has been suggested (Epstein *et al*, 1991; Korber *et al*, 1994; Power *et al*, 1994; Reddy *et al*, 1996; Di Stefano *et al*, 1996; Chang *et al*, 1998). Interestingly, this was a region of absolute identity among our sequences from DWM, BM and monocytes, and served to distinguished them from all others. The V3 sequences from the caudate formed a separate group, different from the other regions of brain. The V3 regions from the other tissues segregated on the basis of tissue, for the most part, except for some commonality between sequences from the LN and lung. These findings support the idea of tissue-based compartmentalization of HIV, and confirm that V3 is an appropriate region to examine in this context. However, caution should be exercised in drawing conclusions based on V3 sequences alone, especially since greater and more meaningful genetic variability may be present within other variable regions, or manifested when the envelope gene is viewed as a whole, as our data show.

We recovered clones with identical patterns of four or more stop codons from different tissues. These clones from different tissues were considerably divergent within the remainder of the gp160 gene, thereby excluding laboratory cross-contamination as an explanation. Close inspection revealed that all of these stops were probably the consequence of G→A hypermutation, a phenomenon frequently observed in the case of HIV (Vartanian *et al*, 1991). The pattern observed suggests that clones CP H7 and men G10, which share four identical stops and diverge by only 1.4% at the

nucleotide level, were the predecessors to the clones CP E4, CP F5 and LN H7, which have nine identical stops, four of which are shared with CP H7 and men G10. How can this be explained, especially since these mutated genes could not produce envelope proteins? The answer is unclear. Of relevance, both the CP and LN can trap and retain HIV particles, in the form of immune complexes, for at least several months, which could provide a mechanism for enhanced longevity of particular viral species, including replication-defective ones (Falangola *et al*, 1994; Heath *et al*, 1995). Also relevant is that these mutations appear to occur in two groups, one spanning 74 amino acids and located at the beginning of gp120, and the other spanning 191 amino acids, with all except the first amino acid positioned within gp41. These mutations could reflect genetic 'hot spots' and thus, have arisen independently within the different tissues. Recombination could account for the diversity present among these clones within the remainder of gp160 (Hu and Temin 1990; Srinivasan *et al*, 1988). Alternatively, their clustering suggests that they could have arisen during just two events, one which generated the first group, and another which generated the second. Hypermutation of several loci within a short region of *env* has been described (Vartanian *et al*, 1991). Since the CP harbored both the 4-stop and 9-stop mutants, it could be the source of the original, 4-stop, mutant, a possibility supported by the relationships shown in the phylogenetic tree. By means of coinfection of the host cell with an HIV strain containing a functional *env* gene, the mutant genome could be packaged into virions with a functional envelope protein and enter new cells within the CP. During the process of reverse transcription in this new cell, the second group of hypermutations could have been generated and the resulting genome, again, by means of coinfection with a competent strain, be packaged into particles. Virions, or infected cells harboring these two defective species could have moved from the CP into the CSF and ultimately, entered the subclavicular lymph node. The CP is a site of infection for many viruses, including HIV, and has been proposed as a route of HIV entry into the CNS (Falangola *et al*, 1995). Also, detection of HIV gp120 in cervical lymph nodes following intraventricular inoculation has recently been demonstrated (Cashion *et al*, 1999). Cross-clade mosiacism has been reported, supporting the contention that different HIV strains can infect the same cell (Salminen *et al*, 1997; Lole *et al*, 1999; Nasioulas *et al*, 1999).

Alternatively, leukocytes harboring the 4-stop mutant could have been circulating within vessels present in the CP and meninges. The one stop codon in meninges D9 at position 692, which is shared with clones CP E4, CP F5 and LN H7, could represent an independent event or be the result of recombination.

The early events leading to the development of HIV dementia are still in question. Increased numbers of macrophages in brain have been shown to correlate with the presence of the clinical disease (Glass *et al*, 1995). This increase could be a consequence of an increase in monocyte trafficking into brain, and/or increased proliferation of resident brain macrophages. Our findings support the first possibility. It is likely that both infected and uninfected monocytes participate in this trafficking, and probable that the majority are uninfected (Shen *et al*, 1994). We propose that this process is either initiated and/or accelerated during late-stage infection, which could explain why dementia occurs primarily during this time.

Materials and methods

Patient information

Patient 546 was a 60-year-old homosexual first identified as HIV positive in 1984. He was followed prospectively in the Multicenter AIDS cohort study. He began experiencing cognitive symptoms, including memory loss and slowed mental processing, in 1991. Impaired reading comprehension and difficulty with wordfinding became apparent later, but overall, his cognitive symptoms were remarkably stable over a number of years. Based on the pattern of his clinical deficits, he was classified as having HIV-associated dementia, stage MSK 1. A sensory neuropathy was also present, which dated back to 1994, as well as a mild extrapyramidal movement disorder with bradykinesia, which first became apparent in 1996. He had been treated with a number of antiretrovirals beginning with zidovudine in 1988. At the time of death he was taking indinavir, Combivir and abacavir. He had had a good virological response on indinavir, his plasma viral load dropping from 1.3 million copies/ml to the undetectable range. In recent months, however, there had been a gradual rise; 3500 copies/ml were present 1 month before death. He died suddenly at home as a consequence of an acute coronary artery thrombosis.

The brain was grossly normal with no generalized atrophy. Diffuse mild perivascular cuffing was found within the white matter of the frontal and occipital cortex and the basal forebrain. There was no evidence of myelin loss using Luxol fast blue staining, and multinucleated giant cells were absent.

Specimen collection and preparation of DNA

Post-mortem tissues were collected approximately 19 h after death. As harvested, tissues were placed in cold RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml of streptomycin. Fresh, disposable forceps and scalpels were used to avoid cross-contamination. The tissues were processed for the preparation of DNA immediately following collec-

tion. Choroid plexus tissues were harvested from the lateral ventricles, and the meninges were collected by delicately teasing them away from larger pieces of cortex and cerebellum. Prior to further processing, all chucks of brain were stripped of any meninges, and all fat and connective tissue was removed from the subclavicular lymph node specimen. All specimens were gently washed in Dulbecco's Phosphate-buffered Saline (PBS) to remove extraneous blood. Fragments of bone marrow were harvested from pieces of sternum and rib. The fragments were dissociated by pipetting, washed in PBS, and the pelleted cells lysed directly. When needed, a disposable Kontes mortar and pestle were used to homogenize the tissues. Lysis buffer containing 50 mM Tris-HCl at pH 7.5, 20 mM EDTA and 0.5% sodium dodecyl sulfate (SDS), and proteinase K at a final concentration of $\sim$400 μg/ml, was added to the tissue homogenates. These preparations were then incubated overnight at 42°C. DNA was extracted using classic phenol-chloroform extraction with RNase digestion. The processing of tissues and preparation of DNAs were carried out in rooms where no plasmid DNA is allowed.

Five months prior to death, a peripheral blood specimen was obtained from patient 546 with informed consent and in compliance with the Human Subjects policies detailed by the National Institutes of Health and the Johns Hopkins University Internal Review Board. Peripheral blood mononuclear cells (PBMC) were obtained by ficoll-hypaque separation and the fresh monocyte fraction purified by adherence for approximately 15 h (Gartner and Popovic, 1990). Such monocyte populations purified in our hands are greater than 95% monocytes, as determined by flow cytometry and staining for nonspecific esterase. Four $\times 10^6$ monocytes were lysed in 100 μl of buffer containing 0.2 M Tris-HCl (pH 7.5), 25 mM EDTA, 0.3 M NaCl and 2% SDS. Proteinase K was added to a final concentration of 650 μg/ml and the lysate incubated at 50°C for 5 h with rocking. The low molecular weight DNA was precipitated using 70% acetone containing 1.5% Dimethylformamide, then pelleted by ultracentrifugation, washed in 70% ethanol, and dissolved in water. The presence of HIV-1 low molecular weight species was confirmed using PCR for the 1- and 2-LTR circular unintegrated forms.

Polymerase chain reaction and molecular cloning
Nested PCR amplification of gp160 genes was performed using the Expand Long kit from Boehringer-Mannheim (Indianapolis, IN, USA), the concentrations of reagents recommended, and a hot start. Reactions were carried out in a 50 μl volume containing $\sim$1 μg DNA, 0.35 μM dNTPs and primers at a final concentration of 0.2 μM. Outer primers were 5′-CTATGGCAGGAAGAAGCGGAGAC-3′ and 5′-TTCTAGCCAGGCACAATCATCATT-3′ and the inner ones were 5′-TAATAGAAAGAGCAGAAGACAGT-3′ and 5′-TTTTGACCACTTGCCACCCAT-3′. The program consisted of an initial denaturation at 94°C for 2 min, followed by 10 cycles of 94°C for 10 s, 55°C for 30 s and 68°C for 4 min. This was followed by an additional 24 cycles as before, but with an additional 20 s added (cumulatively) to the synthesis step of each cycle. A final extension at 72°C for 12 min was included. The second round was performed using identical conditions and 1 μl of the first round reactions. Amplimers were ligated into the pCR3.1 eukaryotic expression vector (Invitrogen Corporation, San Diego, CA, USA) and the ligation reactions were used to transform STBL2 bacterial cells (Life Technologies/Gibco-BRL, Gaithersburg, MD, USA). Colonies were screened for positivity using hybridization and the enhanced chemiluminescence detection method (ECL, Amersham Corporation, Arlington Heights, IL, USA), followed by enzymatic digestion to confirm the length of the viral insert and determine orientation. Measures to guard against cross-contamination included the use of a biological safety cabinet dedicated to PCR amplification of genomic DNA. This hood houses an ultraviolet light and is located within a room where no plasmid DNA is allowed.

Sequencing methods and data analysis
Ultrapure plasmid DNA was prepared using PSI ψ CLONE columns (Princeton Separations, Adelphia, NJ, USA). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 377 sequencer. Three-hundred-and-fifty nanograms of plasmid DNA were used per reaction. The products were purified using Spin-50 mini-columns. Both strands were sequenced using a battery of primers.

The DNAstar software package (DNAstar Inc., Madison, WI, USA) was used to initially align and edit the sequences and determine the consensus for each clone. In all cases, the consensus was based on agreement of overlapping sequences. Each contig and consensus was visually inspected, and all stop codons and frameshifts were reverified. Alignments of the gp160 genes were generated using the Clustal program, and examined and manually refined prior to phylogenetic analysis. Phylogenetic analyses were based on gap-stripped nucleotide sequences and performed using the neighbor-joining method and the Clustal program. Trees were rooted upon the gp160 sequence of the HIV-1 African isolate ELI. Accession numbers for these sequences are AF217150–AF217180.

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