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In Vivo Distribution and Cytopathology of Variants of Human Immunodeficiency Virus Type 1 Showing Restricted Sequence Variability in the V3 Loop

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The distribution, cell tropism, and cytopathology in vivo of human immunodeficiency virus (HIV) was investigated in postmortem tissue samples from a series of HIV-infected individuals who died either of complications associated with AIDS or for unrelated reasons while they were asymptomatic. Proviral sequences were detected at a high copy number in lymphoid tissue of both presymptomatic patients and patients with AIDS, whereas significant infection of nonlymphoid tissue such as that from brains, spinal cords, and lungs was confined to those with AIDS. V3 loop sequences from both groups showed highly restricted sequence variability and a low overall positive charge of the encoded amino acid sequence compared with those of standard laboratory isolates of HIV type 1 (HIV-1). The low charge and the restriction in sequence variability were comparable to those observed with isolates showing a non-syncytium-inducing (NSI) and macrophage-tropic phenotype in vitro. All patients were either exclusively infected (six of seven cases) or predominantly infected (one case) with variants with a predicted NSI/macrophage-tropic phenotype, irrespective of the degree of disease progression. p24 antigen was detected by immunocytochemical staining of paraffin-fixed sections in the germinal centers within lymphoid tissue, although little or no antigen was found in areas of lymph node or spleen containing T lymphocytes from either presymptomatic patients or patients with AIDS. The predominant p24 antigen-expressing cells in the lungs and brains of the patients with AIDS were macrophages and microglia (in brains), frequently forming multinucleated giant cells (syncytia) even though the V3 loop sequences of these variants resembled those of NSI isolates in vitro. These studies indicate that lack of syncytium-forming ability in established T-cell lines does not necessarily predict syncytium-forming ability in primary target cells in vivo. Furthermore, variants of HIV with V3 sequences characteristic of NSI/macrophage-tropic isolates form the predominant population in a range of lymphoid and nonlymphoid tissues in vivo, even in patients with AIDS.

There are several interpretations of the high degree of observed sequence diversity between published sequences of the envelope gene of human immunodeficiency virus type 1 (HIV-1) (subtype B) (54) and of the rapid turnover of HIV envelope variants within infected individuals (69, 79). It has been suggested that changes in the hypervariable domains in the *env* gene (V1 to V5) (52, 70, 78) may facilitate evasion of the host immune system. This conclusion is supported by the observation that V3 and possibly other regions are targets of neutralizing antibodies elicited by natural infection or upon immunization with recombinant gp120 protein (20, 26, 32, 44, 49, 60).

However, changes in the V3 loop, and more recently in the V1 and V2 hypervariable domains, have also been shown to influence the phenotype of variants of HIV-1 in in vitro culture (2, 6, 11, 19, 24, 31, 72, 73). In particular, substitutions of basic amino acids in the V2 and V3 regions change virus isolates from non-syncytium-inducing (NSI) isolates to syncytium-inducing (SI) isolates (11, 19, 24) and may confer a reduction in

the ability of the virus to replicate in macrophages (10, 64). Apart from the association with arginine or lysine at positions 11 and 28 in V3, the SI phenotype has been also shown to correlate with increased V3 sequence heterogeneity in this region (10, 51). V3 sequences from NSI isolates show few sequence differences from each other or from a consensus sequence of 133 North American isolates (40) that comprise predominantly subtype B variants of HIV-1 (46). In contrast, SI isolates show a broad range of substitutions, insertions, and deletions at most positions (30 of a total of 33 to 37) between the disulfide-bridged cysteine residues of the putative V3 loop structure.

In early infection, HIV variants with an NSI/macrophage-tropic (MT) phenotype predominate. Subsequently, approximately half of those individuals who progress to AIDS show a change in virus to a more rapidly replicating (rapid/high) and cytopathic phenotype (3, 8, 18, 74); this switch in phenotype has been reported to precede an accelerated loss of CD4⁺ lymphocytes in the peripheral circulation and a more rapid onset of AIDS than in those individuals whose isolates retain the NSI phenotype (35). However, although most investigators have found a close association between the properties of syncytium induction and inability to replicate in macrophages in vitro and vice versa (10, 12, 19, 23, 37, 63, 64, 87), others

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have found that disease progression is associated with a switch from NSI, non-MT isolates to SI/MT isolates (8).

Despite their more aggressive phenotype *in vitro*, the SI isolates are less transmissible by sexual contact than are NSI isolates (59, 87), but they are equally transmissible in cases of mother-to-child transmission (62). To explain these apparently contradictory findings, it has been speculated that macrophages may be the first cells infected at the mucosal barrier in the case of sexual transmission and that infection of this cell type is responsible for the systemic dissemination of the virus found upon primary infection (87). However, V3 sequences typical of NSI isolates were also found to be specifically selected for not only upon sexual contact but also in several cases of parenteral infection (hemophiliacs exposed to HIV-contaminated factor VIII concentrate) in which case the initially infected cells could equally well be CD4⁺ lymphocytes (85).

An important limitation of many of the previous studies of sequence and phenotype change of HIV-1 is the reliance upon patient blood samples as sources of virus isolates, proviral DNA, or virion RNA as study material. However, HIV results in systemic disease and has been shown to be capable of infecting a wide variety of nonlymphoid tissue, including tissue from brain, lung, and the small and large bowel (reviewed in reference 41). It cannot be assumed that variants in blood are representative of populations infecting other cell types in the body. For example, isolates from brain and bowel tissues show *in vitro* properties different from those derived from peripheral blood mononuclear cells (PBMCs); the latter tend to replicate well in macrophages and to show an NSI phenotype (25, 36, 76). In some cases, sequence differences between populations of variants in brain tissue and those in circulating lymphoid cells have been observed (16, 25, 33, 42, 43, 57, 71). The extent to which sequence changes are responsible for the differences *in vivo* tropism is discussed below.

Given the evidence for changes in the *in vitro* phenotype of virus isolated from PBMCs at different stages of disease progression, it is clearly important to investigate whether equivalent changes occur in virus populations outside the peripheral circulation. By carrying out detailed quantitative studies of postmortem tissue, we have recently found a highly restricted distribution of HIV in the body preceding the onset of AIDS, with proviral sequences apparently confined to cells of the lymphoid system (PBMCs, spleen, and lymph nodes). In contrast, those patients who died from complications associated with AIDS showed significant infection of cells in the central nervous system (CNS) and in lung and bowel tissues (13). These results confirm a previous study in which HIV infection was undetectable in the CNS of a large number of individuals who died while they were asymptomatic (5).

This apparent redistribution of virus upon disease progression occurs at the same stage of disease as the change from an NSI to an SI phenotype. Paradoxically, while isolates become cytopathic and often non-MT *in vitro*, the redistribution of HIV *in vivo* involves organs such as brains and lungs and other tissues in which the main targets of infection are reported to be tissue macrophages, microglia (in the CNS), and other non-lymphocyte cell types (22, 34, 47, 55, 77).

In this study, we have carried out detailed sequence comparisons of the V3 loop and flanking regions of virus variants in lymphoid and nonlymphoid tissues from a series of individuals who died while asymptomatic or as a consequence of terminal AIDS. The inferred *in vitro* phenotypes were compared for individuals with differing degrees of disease progression and for different tissue types in order to explore the relationship between V3 variation and tissue tropism of HIV *in*

vivo. In many cases it was possible to directly identify the target cells and degrees of associated cytopathology in different tissues (*in vivo* phenotype) by immunocytochemical staining of the tissues with an anti-p24 monoclonal antibody.

MATERIALS AND METHODS

Patient samples. Cardiac blood and tissue from various organs were obtained within 3 days of patient death from 11 autopsied HIV-infected patients of whom three (patients 1 to 3) died suddenly while they were in the asymptomatic stage of HIV infection as defined by Centers for Disease Control criteria and four patients (patients 4 through 6 and patient 9) who died with AIDS-defining illness. Additional lymph node samples were obtained from four further patients who died of complications associated with AIDS (patients 10 through 13) for further sequence comparisons of sequences in the V3 and *gag* regions. Clinical information and laboratory investigations pertaining to patients 1 to 6 have been detailed as part of an earlier study (13). Patient 9 was a 59-year-old male with a recent history of psychomotor slowing. One week prior to death he developed a fatal atypical pneumonia, at which time a diagnosis of full-blown AIDS was made. He received no antiviral treatment, and the duration of his infection is unknown.

Samples of brain (left frontal lobe), spinal cord (mid-thoracic), lung, large bowel, mesenteric lymph node, and spleen tissues from these patients were dissected into 1- to 2-cm pieces and were stored at -70°C . DNA was extracted from tissues and whole blood to obtain total DNA from all nucleated cells in the circulation (peripheral blood nucleated cells [PBNCs]) as previously described (68).

Quantitation. Proviral DNA was quantified by using a previously described limiting dilution and nested-primer PCR approach (68). The quantitation was performed in the first instance by using primers corresponding to the *pol* gene and was performed subsequently with primers spanning the V3 region. The nucleotide sequences of the primers and the position of the 5' base in the HXB2 genome (54) were *pol* a CATGGGTACCAGCACACCGG, + (sense), position 4149; *pol* b GGAGGAAATGAACAAGTAGATA, +, 4175; *pol* c TCCTAGCCATTGCTCTCCAATT, - (antisense), 4290; *pol* d TCTACTGTGCCATGCATGGCTTC, -, 4380; V3 e TA CAATGTACACATGGAATT, +, 6957; V3 f TGGCAG TCTAGCAGAAGAAG, +, 7009; V3 g CTGGGTCCCCTC CTGAGG, -, 7331; and V3 h ATTACAGTAGAAAAATTC CCC, -, 7381.

All reactions were performed with appropriate positive and negative controls. The number of proviral copies was estimated by assuming a Poisson distribution for each sample by $-\log(1-p)/d$ (where p = proportion of positive samples and d = dilution). Likelihood ratio tests were used to determine whether there were significant differences between sample pairs. The likelihood function is proportional to $[1 - \exp(-\lambda d)]^m \times [\exp(-\lambda d)]^n$, where λ = number of proviral copies, m = number of positive replicates, and n = number of negative replicates. Ninety-five percent confidence intervals for each sample were determined by evaluating the likelihood function incrementally over the range of possible values for the number of proviral copies. The overall level of agreement between the *pol* and *env* primers was assessed by using the paired t test on the basis of the numbers of proviral copies on a log scale.

Sequence analysis. Single molecules of HIV-1 provirus were isolated by limiting dilution and amplified in a nested PCR to produce sufficient DNA to allow direct sequencing of the PCR products. Direct sequencing of amplified DNA was achieved

TABLE 1. Quantitation of proviral sequences in postmortem tissue by limiting dilution using nested primers in the *pol* and V3 regions: frequency of positive results at specified dilutions of DNA

Patient	Primer ^a	Frequency ^b of positive results						
		Lymphoid tissue			Nonlymphoid tissue			
		Lymph node	Spleen	PBNCs	Brain	Spinal cord	Lung	Colon
Presymptomatic								
1	E	9/20 (1E-3) ^c	4/20 (1E-3)	12/20 (1E-2)	0/20 (5E-1)	3/20 (5E-1)	0/20 (5E-1)	0/20 (5E-1)
	P	19/60 (5E-4)	ND ^d	18/25 (2E-2)	ND	ND	ND	ND
2	E	14/20 (1E-3)	12/20 (1E-2)	7/20 (1E-3)	2/20 (5E-1)	15/20 (5E-1)	6/20 (1E-1)	10/20 (1E-1)
	P	ND	ND	20/50 (2E-3)	ND	ND	ND	ND
3	E	9/20 (1E-1)	7/20 (1E-2)	9/20 (1E-2)	3/20 (5E-1)	3/20 (5E-1)	3/20 (1E-1)	6/20 (5E-1)
	P	2/20 (5E-2)	ND	8/30 (1E-2)	ND	ND	ND	ND
Symptomatic								
4	E	14/20 (5E-3)	3/20 (1E-3)	5/20 (1E-2)	3/20 (1E-2)	7/20 (1E-2)	18/20 (1E-2)	17/20 (1E-2)
	P	19/30 (6E-3)	16/30 (5E-3)	21/50 (5E-2)	17/50 (2E-2)	10/25 (1E-2)	14/49 (2E-3)	18/30 (6E-3)
5	E	17/20 (1E-3)	4/20 (1E-3)	7/20 (1E-1)	4/20 (1E-3)	11/20 (1E-4)	17/20 (5E-3)	7/20 (1E-1)
	P	41/50 (1E-3)	ND	23/50 (1.5E-1)	16/50 (1E-3)	ND	14/50 (8E-4)	ND
6	E	15/20 (1E-2)	19/20 (1E-2)	4/20 (5E-1)	12/20 (1E-2)	10/20 (1E-1)	11/20 (1E-1)	11/20 (1E-3)
	P	23/50 (5E-3)	ND	1/20 (5E-2)	14/50 (4E-3)	ND	ND	12/50 (8E-4)
9	E	7/50 (2E-2)	ND	ND	22/49 (2.5E-4)	ND	ND	ND
	P	ND	ND	03/ (1) ^c	ND	2/3 (1E-2) ^c	0/3 (1) ^c	0/3 (1) ^c

^a E, *env* primer quantitation; P, *pol* primer quantitation.^b Expressed as number positive/total number.^c Test dilution (in micrograms); 1E-3 = 1×10^{-3} µg.^d ND, not done.^e p24^{gag} quantitation.

either as previously described or by using a solid-phase sequencing method (30). For solid-phase sequencing, the second PCR reaction was performed in a 100-µl volume by using one biotin-labelled and one unlabelled primer (5 to 10 pmol of primer per reaction mixture), generating a PCR product with one strand having a biotin moiety at the 5' end. PCR products were immobilized on streptavidin-coated magnetic beads (Dyna), and single strands of DNA were purified by magnetic separation and were sequenced according to the manufacturer's protocol.

A Fisher's exact test for $2 \times n$ contingency tables (48) was used to compare amino acid frequencies at each position in the V3 loop (n is the number of alternative amino acids at a single position). The test is based on exact multinomial probabilities and is appropriate here because many values in the contingency tables were small. Comparisons of V3 loop charge and diversity were made between sequences obtained in this study and those of a series of isolates of HIV-1 of known biological properties. V3 loop sequences of 30 isolates with an SI phenotype and 29 NSI/MT isolates were obtained from references 10 and 19, as were corresponding sequences from MN, RF, SF2, GUN-1, HAN-2, SF33, LAI, ADA, YU2, SF162, JF-L, and SF-128A isolates (51, 54).

Evolutionary analysis of p17 sequences. Sequence comparisons between viruses from the 11 study patients were made in the region of the *gag* gene encoding p17, as previously described (29). Phylogenetic relationships between single nucleotide sequences from each of the study patients and from representative sequences obtained both in Edinburgh, United Kingdom (29a), and from other widely separated geographical localities (54) were estimated by using the neighbor joining method with a bootstrap resampling program (PHYLIP programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE) (17). Branch lengths on this tree were estimated by using the maximum-likelihood method (DNAML). Nucleotide distances between sequences were estimated by using the substitution model of Felsenstein (program DNADIST).

Immunocytochemical staining. Five-micrometer sections of formalin-fixed paraffin-embedded tissue from the study organs were stained with hematoxylin and eosin by standard methods. p24 antigen was detected by an avidin/biotin immunocytochemical technique (5) with a monoclonal antibody to p24 (Dupont) as first antibody at a dilution of 1/200 and diaminobenzidine as the visualizing agent. Prior to antibody incubations, the sections were irradiated in an 800-W microwave oven for three cycles of 5 min each while they were immersed in 0.01 M citrate buffer (pH 6.0) (Fisons).

Nucleotide sequence accession numbers. Sequences obtained in this study have been deposited into GenBank and have accession numbers L34422 to L34541.

RESULTS

Quantitation of proviral load in different organs. The numbers of copies of provirus per million cells in a range of lymphoid and nonlymphoid tissues from the study patients were determined by limiting-dilution PCR with primers from both *pol* and *env* regions of the genome (Table 1; results obtained with the *pol* primers for patients 1 to 6 have been previously reported) (13). We found high levels of HIV in lymphoid organs (spleen and lymph node) from all presymptomatic and symptomatic individuals with both sets of primers, whereas nonlymphoid organs were infected only in patients who died of complications associated with AIDS. Although low levels of provirus were found in many of the nonlymphoid tissues of the presymptomatic patients (up to 46 copies per 10^6 cells; Table 2), such virus may have originated from PBNCs in residual blood within the organs (forming between 1 and 10% of the extracted DNA from the tissue, depending on its vascularity), as previously discussed (13). For example, the sample of colon tissue from patient 2 contained 46 copies of provirus per 10^6 cells, 60 times lower than the frequency of infected cells in peripheral blood (2,843 copies per 10^6 PBNCs) and attributable to the presence of peripheral blood in the

TABLE 2. Quantitation of proviral sequences in postmortem tissue by limiting dilution using nested primers in the *pol* and V3 regions: calculated proviral copies per 10⁶ cells

Patient	Primer ^a	No. of copies (confidence interval ^b)						
		Lymphoid tissue		Nonlymphoid tissue				
		Lymph node	Spleen	PBNCs	Brain	Spinal cord	Lung	Colon
Presymptomatic								
1	E	3946 (2092-7616)	1472 (601-3788)	337 (172-673)	0 ^c (0-2.44)	2.18 ^c (0.79-6.27)	0 ^c (0-2.44)	0 ^c (0-2.44)
	P	5026 (3227-7880)	ND ^d	420 (264-700)	ND	ND	ND	ND
2	E	7946 (4693-14051)	605 (343-1089)	2843 (1399-5920)	1.39 ^c (0.46-5.02)	18 ^c 11-32	23 ^c 11-51	46 ^c 24-64
	P	ND	ND	1686 (1096-2620)	ND	ND	ND	ND
3	E	39 ^c (21-63)	284 (139-594)	394 (211-759)	2.18 ^c (0.79-6.27)	2.18 ^c (0.79-6.27)	11 ^c (4-31)	4.69 ^c (2.18-10.36)
	P	14 ^c (4-49)	ND	205 (106-403)	ND	ND	ND	ND
Symptomatic								
4	E	1589 (937-2811)	1073 (389-3141)	190 (86-449)	107 (40-317)	284 (139-594)	1520 (917-2897)	1252 (759-2270)
	P	1103 (700-1775)	1006 (620-1663)	72 (46-112)	137 (86-218)	337 (185-627)	1110 (667-1868)	1009 (634-1630)
5	E	12520 (7583-22704)	1473 (601-3788)	28 (13-57)	1473 (601-3782)	52701 (27997-65049)	2504 (1518-4541)	28 (14-57)
	P	11317 (8144-16210)	ND	27 (28-40)	2545 (1570-4151)	ND	2710 (1624-4567)	ND
6	E	915 (548-1617)	1977 (1175-4402)	2.97 (1.19-7.59)	604 (343-1089)	46 (24-64)	53 (28-65)	5270 (2950-9682)
	P	813 (541-1234)	ND	6.80 (1.65-37.55)	542 (323-911)	ND	ND	2264 (2086-6349)
9	E	49 (23-65)	ND	ND	15733 (12988-30049)	ND	ND	ND
	P	ND	ND	0 (0-8.05)	ND	725 (231-3155)	0 (0-8.05)	0 (0-8.05)

^a E, *env* primer quantitation; P, *pol* primer quantitation.^b Ninety-five percent.^c Below 1/10 of value in PBNC, accounted for by residual blood in the organ.^d ND, not done.

postmortem tissue. In contrast, the majority of organs from the patients with AIDS showed high levels of provirus, consistent with actual infection of cells within the tissue by HIV. Only samples with clear evidence of infection by these criteria were used for comparisons of sequences in the *env* gene (see below).

The accuracy of quantitation by limiting dilution is dependent on the number of replicates tested and the frequency of positive reactions. We calculated the confidence intervals for quantitation with *pol* and *env* primers by evaluating the likelihood function for each sample (see Materials and Methods) and found that there was no statistically significant difference between the primers for any of those tested (Table 2). When the samples were taken together, the frequency of provirus quantified with *env* and *pol* primers showed a ratio of 1.18 (confidence interval, 0.94 to 1.48; Fig. 1). The correlation coefficient for quantitation with the two sets of primers was 0.96 ($P = 0.0001$), and there was no evidence for an over- or underrepresentation of *env* or *pol* sequences in either lymphoid or nonlymphoid tissue. These results indicate that the V3 primers are as efficient as *pol* primers in amplifying HIV and that we did not amplify only a specific subset of variants in our subsequent sequence comparisons (see Discussion).

Sequence variation in the V3 loop. DNA extracted from different organs or from PBNCs was diluted until only 10 to 20% of replicates gave a PCR product, thus ensuring that nucleotide sequences were derived from single molecules of provirus (68). The lack of peripheral infection in the asymptomatic group restricted our sequence comparisons to virus within lymphoid tissue only (PBNCs and lymph node). For the patients who died of complications associated with AIDS, we were able to carry out more extensive comparisons with a range of samples from nonlymphoid tissues, such as those from brain and lung tissues (Table 2). None of the 322 nucleotide sequences in the V3 loop or flanking regions contained inactivating substitutions such as stop codons or frameshifts. Only one sequence (from lymph node tissue from patient 3) showed G→A hypermutation, producing a highly unusual and probably nonfunctional provirus. Like previous researchers who used the limiting-dilution/direct-sequencing method of sequence determination (4, 68, 85), we have found no evidence for high rates of defective genomes in vivo.

In the asymptomatic patients, a wide range of sequence variants were found in both PBNCs and lymph nodes (Fig. 2). In patients 1 and 3 there were statistically significant differences in the frequency of major and minor amino acid sequences present in the two types of sample. In these two patients, the majority form, in the PBNCs, differed from that in the lymph nodes and vice versa, although in each case such variants were present as minority components in the other sample.

There was a similar diversity of V3 sequences as well as of population differences between samples from the patients who died of complications associated with AIDS. Extreme sequence diversity in all samples from patient 4 was observed, while samples from patient 9 were restricted to only two different sequences in the brain and to three in the lymph nodes. As with the presymptomatic patients, the frequencies of different variants between some organs were statistically significant, although a general observation was of a common set of sequences being present at varying frequencies throughout the body. For example, for patient 4, the main sequence in the brain (12 of 17) and spinal cord (6 of 7) also occurred in the colon (5 of 16), lung (7 of 15), and lymph nodes (1 of 16). However, there are also variants that appear to be more restricted in distribution (e.g., the colon of patient 4, the brain of patient 5, and the colon of patient 6). This type of analysis

provides no evidence for the existence of a shared determinant in the V3 loop that governs the distribution of HIV variants. This problem is compounded by not knowing whether the different sequences within an organ represent genuine diversity of HIV in a single cell type, or whether they result from the presence within the same organ of various proportions of several different HIV-infected cell types, each bearing different proviral variants (such as CD4⁺ lymphocytes, dendritic cells, and macrophages in the lymph nodes).

To investigate in more detail the process of sequence diversification of the V3 loop upon disease progression, we compared the number of different V3 amino acid sequences found within samples from the presymptomatic patient group and from the group of patients with AIDS (Table 3). In both cases, frequent nonidentical sequences were found; the presymptomatic patients showed a total of 21 different sequences of 84 sampled, compared with 18 of 92 in lymphoid tissue and 23 of 146 from nonlymphoid tissue in the patients with AIDS. Similarly, the mean evolutionary distances between nucleotide sequences in the presymptomatic group (0.042) differed little from distances between sequences found in the patients with AIDS (0.038; Table 4). These comparisons also show that little difference in diversity exists between populations of HIV infecting lymphoid tissue and those replicating in nonlymphoid tissue such as that from the brain, lung, and colon (Tables 3 and 4).

Prediction of in vitro phenotype from V3 loop sequences.

There exist well-defined relationships between the properties of macrophage tropism and syncytium induction on the one hand, and between the net V3 charge (11, 19) and similarity to the subtype B consensus V3 sequence (10) on the other hand. Comparison of the 54 different V3 loop sequences produced a consensus sequence that was identical in all but one position to the subtype B consensus (40). There was a striking similarity between this set of sequences and a sequence set of 29 isolates characterized in vitro as showing an MT and NSI phenotype, not only in overall consensus sequence but also in the position and nature of amino acid substitutions that did occur (Fig. 3). For example, both data sets contain frequent substitutions of asparagine (N) and proline (P) for histidine (H) at position 15 and contain either aspartate (D) or glutamate (E) at position 29, with frequent substitutions of alanine (A) or glutamine (Q).

In contrast, V3 loop sequences from a collection of SI sequences were highly variable and contained a number of amino acid replacements not found in the other sequences. For example, residues 7 to 9 are invariant among the postmortem and NSI/MT sequences but are highly polymorphic in the SI isolates. Other residues such as that at position 12 show differences of the consensus sequence (serine [S] in postmortem and NSI/MT sequences and arginine [R] in the SI variants). To investigate whether the postmortem sequences were significantly more similar to those of MT isolates, we carried out Fisher's exact tests (see Materials and Methods) at each amino acid position in the V3 loop. Results at each amino acid position are not independent, so statistical results have to be interpreted cautiously; values are listed only when P is less than 0.01. These tests showed that between postmortem and NSI/MT isolates, there were significant differences in the populations of amino acids only at positions 16 ($P \approx 0.003$) and 22 ($P \approx 10^{-4}$) (Fig. 4). In contrast, postmortem sequences differed significantly at several positions from the SI variants, the most divergent residues being 10 ($P < 10^{-8}$), 12 ($P < 10^{-8}$), and 29 ($P < 10^{-6}$). This statistical evidence supports the previously observed association between the presence of basic (arginine or lysine) residues at positions 12 and 29 and the SI phenotype (19), although other types of substitutions at these

FIG. 1. Comparison of quantitation of provirus by using *pol* and V3 primers by limiting dilution (log scales). O, samples extracted from lymphoid tissue (lymph node, spleen, and PBNCs); ●, samples extracted from brain, spinal cord, lung, and colon.

sites also occur specifically in the SI variants. There are also further positions at which SI variants differ considerably from postmortem and NSI/MT variants (positions 7 to 10, 15, 28, and 36).

To investigate the relationship between in vitro phenotype and V3 sequence, we have calculated the overall V3 charge and the degree of sequence divergence from the subtype B consensus for a series of isolates with known biological properties (Fig. 5a). In agreement with a previous report which used a similar method for sequence analysis of V3 (51), NSI/MT isolates consistently showed lower charge, greater similarity to the subtype B consensus, or both, than did SI and non-MT variants. A diagonal line almost completely separates the two populations. Using this analysis to predict the phenotype of variants found in postmortem tissue of presymptomatic and terminal patients with AIDS, we found that almost all sequences were located to the left of the dividing line and that they could therefore be predicted to be of the NSI/MT phenotype (Fig. 5b and c). Indeed, there was a tendency for some of the postmortem sequences, particularly those from nonlymphoid tissue, to show a lower charge and fewer differences from the subtype B consensus than did the "typical" NSI isolate. The only sequences with overall charge and divergence approaching that of SI variants were some of those found in patients 3 and 9.

Although the sequences obtained in this study conform closely to those previously described for MT variants in vitro, it is possible that the restriction in sequence diversity in V3 was, at least in part, the result of sampling a population of individuals infected with a very limited subset of HIV-1 variants. To investigate this possibility, we carried out sequence comparisons in the p17^{arg} for all of the 11 study patients. Phylogenetic analysis of sequences in the p17 region have been shown to provide a reliable indication of epidemiological relationships between variants within the same subtype B of HIV-1 (27, 29).

FIG. 2. Proviral V3 loop amino acid sequences from infected organs from asymptomatic patients (1 [p1] through 3) and from patients with terminal AIDS (patients 4 through 6 and patient 9). All sequences are compared with subtype B consensus sequence for V3 (40); a period indicates identity with subtype B consensus; minus indicates a gap introduced to preserve alignment with consensus sequence. n, number of sequences observed.

TABLE 3. Comparison of sequence variability in the V3 loop upon disease progression: frequency of different sequences

Patient type	No. of sequences					
	Lymphoid tissue		Nonlymphoid tissue		Total	
	Total	Unique	Total	Unique	Total	Unique
Presymptomatic	84	21			84	21
Symptomatic	92	18	146	23	238	31
Total	176	36	146	23	322	47

No direct epidemiological relationship was found between viruses infecting the 11 study subjects (Fig. 6). Although many of the subjects were infected with variants that fell within the main (heterosexual and intravenous-drug user) cluster of Edinburgh patients, there was no evidence of direct epidemiological contact between them. Furthermore, several variants were separated by those of published isolates of HIV-1 from North America and elsewhere, suggesting a distant evolutionary relationship between them. This analysis confirms the clinical impression that the patients in this study were unrelated epidemiologically to each other. However, there was no phylogenetic information within the V3 region of the *env* gene: sequences showed at most six amino acid changes (patient 9) from the subtype B consensus sequence. All but one V3 sequence (patient 10) is predicted to have an in vitro NSI/MT phenotype according to the analysis presented in Fig. 5. Although certain V3 sequences are more divergent than others, this does not correlate with the underlying phylogenetic relationships between the corresponding p17^{gag} sequences within the HIV-1 B subtype.

TABLE 4. Comparison of sequence variability in the V3 loop upon disease progression: mean sequence diversity

Patient type	Sequence diversity ^a		
	Lymphoid tissue	Nonlymphoid tissue	Total
Presymptomatic	0.042		0.042
Symptomatic	0.038	0.039	0.0385
Total	0.040	0.039	0.040

^a Mean evolutionary distance between nucleotide sequences.

Localization and cytopathology of HIV-1 in vivo. None of the previous investigations described in this study identify the cells infected with HIV. It is therefore not clear whether the

predicted macrophage tropism and NSI phenotype of the postmortem sequences reflects the tropism and cytopathology of HIV-1 *in vivo*. To show this, we performed immunocytochemical detection of p24 antigen to localize the infected cells in the tissues for which we carried out the sequence analysis.

For nonlymphoid tissue, we found a very good agreement between the detection of p24 antigen and virus load as determined by limiting-dilution PCR (Table 5). No p24 was found in nonlymphoid tissue of any of the presymptomatic patients, while antigen-expressing cells were frequently found in the brains, spinal cords, and lungs of several of the patients with AIDS (Tables 1 and 5). For example, patient 5 showed high levels of provirus and p24 antigen in several organs, while antigen-expressing cells in patient 6 were confined to the brain.

The lymph node architecture of the presymptomatic patients was relatively normal or showed hyperplasia (Fig. 7a), with none of the evident T-cell depletion, involution, or fibrosis which is found in the lymphoid tissue of patients with AIDS. The interpretation of p24 antigen staining in lymph nodes and spleen was complicated by the presence of extracellular virions captured on the surface of follicular dendritic cells in the B-cell areas (Fig. 7b) (15, 58). p24 antigen was confined to cellular processes of what morphologically appeared to be follicular

POST MORTEM VARIANTS

[illegible]

NSI / MACROPHAGE-TROPIC ISOLATES

[illegible]

SI / T-CELL LINE-TROPIC ISOLATES

[illegible]

FIG. 3. Comparison of sequences obtained in this study from postmortem material (54 different V3 loop sequences from a total of 322) with those of isolates of HIV-1 showing an NSI and MT phenotype ($n = 29$), and isolates that are SI and non-MT ($n = 30$). Position and frequency (in subscript) of specific substitutions are indicated below the consensus. See Materials and Methods for sources of sequences.

FIG. 4. Comparison of the distributions of amino acid changes at each position in the V3 loop between postmortem sequences and those of NSI/MT isolates (solid line) and those of SI/T-cell-tropic isolates (dotted line) in vitro. The probability of the distribution of changes arising by chance is indicated on the y axis on a log scale. Sequences compared correspond to those shown in Fig. 3.

dendritic cells and was not found in the T-cell areas of either presymptomatic or terminal patients with AIDS (Fig. 7b). The failure to detect p24 antigen staining outside the lymphoid follicles suggests that the provirus-bearing cells in lymphoid tissue are largely transcriptionally inactive, although the p24 assay used in this study may not be able to detect low-level expression of virus proteins.

Elsewhere, p24 antigen-expressing cells in vivo were found in brain (Fig. 7c and d), spinal cord, and lung tissue (Fig. 7f and h). In brain and spinal cord tissues of patients with AIDS, p24 antigen was detected in multinucleated giant cells (Fig. 7c), in mononuclear macrophages, and frequently in morphologically normal microglial cells (Fig. 7d). In general, the presence of p24 antigen-positive cells was associated topographically with evidence of tissue damage.

In the lung tissue of patient 4, frequent p24 antigen-expressing lung macrophages were found, and, as in the brain, infected cells formed pronounced multinucleated syncytia (Fig. 7f). For this patient, the predominant V3 sequence of provirus amplified from lung tissue was identical to the major variants infecting the brain and spinal cord and to a proportion of those in colon tissue (Fig. 2). However, this sequence was not represented significantly among sequences of provirus infecting lymphoid tissue from the same patient. The other patient who by quantitative PCR (patient 5; Table 1) showed significant infection of the lungs showed a sharply contrasting tissue distribution of infection. Pathological examination of the lung revealed prominent lymphocytic infiltration into lung tissue and formation of poorly formed lymphoid follicles adjacent to bronchioles (Fig. 7g and h). p24 antigen was detected within the lymphoid follicles but not elsewhere in the lung despite the presence of some multinucleated macrophages. In this patient the population of V3 sequences in lung tissue corresponded closely to that in lymphoid tissue and was distinct from that of provirus variants infecting the brain, where infection of macrophages and microglial cells was prominent (Fig. 2).

DISCUSSION

Restricted sequence variability in the V3 region. An unanticipated finding in this study was the limited sequence variability in the V3 loop of HIV amplified from tissues in vivo. This restriction was evident irrespective of the degree of

FIG. 5. Comparison of V3 loop sequences on the basis of predicted overall charge (x axis) and number of amino acid changes from the subtype B consensus (40) (y axis). (a) Fifty-nine isolates of known phenotype in vitro; ○, NSI and MT; ●, SI, non-MT. See legend to Fig. 3 for sources of sequences. A diagonal line separates the two populations. Equivalent analysis was carried out upon the nonidentical V3 loop sequences collected postmortem from three presymptomatic individuals (b) and four patients who died from complications associated with AIDS (c). Origins of sequences: ○, PBNCs; □, lymph nodes; +, brain; ◆, spinal cord; ●, lung; ■, colon. The V3 loop charge was calculated by assigning a unitary positive charge to arginine and lysine residues and a unitary negative charge to glutamate and aspartate residues. The potential partial charge contributed by histidine residues was discounted.

TABLE 5. Detection^a of p24 antigen in postmortem tissue

Patient	Staining result				
	Lymphoid tissue		Nonlymphoid tissue		
	Lymph node	Spleen	Brain	Spinal cord	Lung
Asymptomatic					
1	+	+	—	—	—
2	+++	+++	—	—	—
3	ND ^b	—	—	—	—
Symptomatic					
4	ND	—	++	++	+++
5	+++	++	+++	+++	++
6	—	—	++	—	—
9	ND	++	+++	—	—

^a Graded from — (negative) to +++ (extensive staining, frequent antigen-expressing cells).

^b ND, not done.

disease progression and tissue origin, whether lymphoid or nonlymphoid. Both the consensus and observed polymorphisms almost exactly matched those found in a separate analysis of isolates showing an MT/NSI phenotype *in vitro*, but were quite distinct from those of SI variants (Fig. 4). The infrequency with which variants with a predicted SI phenotype were detected *in vivo* contrasts with their frequent isolation from patients upon disease progression (3, 8, 18, 74). For example, among the four patients with AIDS in this study, only two variants were found to show sufficient positive charge and divergence from the consensus sequence to place over the

dividing line that separates isolates with different properties (Fig. 5c).

We were able to rule out two possible sources of bias in our results, i.e., that the primers used for amplification of the V3 loop preferentially amplified NSI variants, and that we were studying an epidemiologically very restricted group of patients. To address the first possibility, we compared quantitation of proviral sequences by using primers specific for both V3 and the well conserved *pol* region. Evaluating the likelihood function to determine confidence intervals for the quantitation method used in this study, we found no significant differences between virus loads when we used the two sets of PCR primers. These data make it unlikely that we failed to amplify a significant proportion of envelope sequences and, indeed, the close concordance between the results for *pol* and V3 in every sample further suggests that each of the sequences analyzed was derived from a complete provirus (see below).

The second potential bias was addressed by phylogenetic analysis of p17^{gag} sequences from the 11 patients from whom we obtained V3 loop sequences. This analysis suggested that the individuals were infected with a representative range of HIV-1 subtype B variants and that the similarities observed in the V3 loop did not result simply from infection with relatively homogeneous and possibly epidemiologically related variants.

The high frequency of variants with a predicted NSI phenotype is not inconsistent with the results of previous studies. Variants with predicted NSI phenotypes were frequently detected in several published analyses of viral sequences *in vivo*, in many cases from patients with advanced HIV-related disease (16, 28, 33, 37, 51, 56, 62, 67, 80, 81, 86), although their frequency relative to SI variants varies considerably between patient groups. Sequence comparisons of the V3 loop of

FIG. 6. Phylogenetic analysis of sequences in the p17^{gag} gene of the study patients, and comparison with V3 loop sequence diversity. (Left panel) Phylogenetic relationships represented by a rooted tree, with the HIV-1 subtype D sequence ELI as an outgroup. ○, study patients; ●, other HIV-infected Edinburgh patients. (Right panel) Majority amino acid sequence from PBNCs (P) or lymph node (L) of samples from study patients in the V3 loop; only differences from the subtype B consensus sequences (40) are shown (see legend to Fig. 2).

FIG. 7. Morphology and immunocytochemical detection of p24 antigen in lymphoid and nonlymphoid tissue. (a) Lymph node of patient 2 (asymptomatic) at low magnification ($\times 31$); hematoxylin and eosin stain. Normal germinal centers are set in the lymphocyte cortex (arrows). (b) High-magnification ($\times 193$) view of the same lymph node stained for p24 antigen, showing association of viral protein with follicular dendritic cells. (c) High-magnification view of section of frontal lobe of the brain from patient 5, stained for p24 antigen, showing prominent virus expression in giant (syncytial) cells (nuclei indicated by arrows; $\times 308$). (d) Same case showing p24 positivity in cells of microglial morphology (arrows; $\times 193$). (e) Section of lung from patient 4 showing frequent macrophage syncytia (arrows) in lung alveoli filled with mononuclear macrophages and edema

sequential samples collected upon disease progression often show a change from a predicted NSI to SI phenotype (80), although there is no evidence for a complete replacement. For example, in an HIV-infected hemophiliac monitored over a period of 6 years, V3 loop sequences were NSI in early infection and became predominantly SI between 4 and 5 years after infection but subsequently reverted to an apparent NSI phenotype (28, 69).

The observed restriction in sequence variability of the V3 loop can be plausibly accounted for by strong selection against sequence change in this region, although with some tolerance of certain amino acid replacements at specific sites (28, 51). The mechanism of selection for these variants remains obscure (see below), but it is significant that exactly the same restriction in sequence diversity in the V3 loop in variants associated with primary infection has been observed *in vivo* (38, 85, 87).

In vitro phenotype of HIV. Although we have not been able to confirm the phenotype of the postmortem variants experimentally, direct evidence that variants found in the CNS were indeed MT has been reported by others (9, 33). In one study, it was found that almost all isolates derived from the cerebrospinal fluid from a range of asymptomatic and symptomatic individuals were capable of efficient replication in primary macrophage culture (33); these variants showed V3 loop sequences with low charge and little divergence, if any, from the subtype B consensus.

Most of the published sequences of SI variants used for sequence comparisons were derived from isolates of HIV-1 that were often passaged extensively in cell culture prior to biological characterization. It is possible that whatever selective constraint restricts sequence diversity *in vivo* is absent in the conditions used for virus culture and that the virus is therefore free to drift away from the subtype B consensus sequence. It is also possible that a V3 (or V2) loop with a large positive charge confers a growth advantage *in vitro*, leading to the selective isolation of variants bearing such divergent sequences from a heterogeneous *in vivo* population. Indeed, the isolation of SI variants from patients progressing to AIDS is associated with increased virus load and therefore with a greater likelihood that such extreme variants might by chance be present in the initial PBMC culture.

Changes in the properties of isolates upon *in vitro* passaging are commonly observed. Repeated passaging enables HIV to adapt to efficient replication in different cell types, including permanently transformed T-cell lines. It has also been shown that *in vitro* culture leads to a rapid loss of sequence variability in the *env* gene (39) and often to the replacement of the predominant *in vivo* variant with a minor population (37, 39, 50). Indeed, specific outgrowth of SI variants has been found upon short-term primary lymphocyte culture of PBMCs with V3 loop sequences that could be predicted to be mainly NSI/MT (37, 47a, 61). These data are consistent with the hypothesis that the overrepresentation of SI isolates from patients with AIDS compared with their frequency *in vivo* is at least in part the direct result of their competitive advantage over NSI/MT variants in certain *in vitro* culture conditions and therefore does not necessarily reflect their prevalence *in vivo*.

Other lentiviruses such as HIV-2 and several of the simian immunodeficiency viruses (SIV) have envelope proteins with many structural similarities to HIV-1 gp160. In particular, it is

possible to identify the homologs of the V1/V2 and V4 hypervariable regions in these different viruses. One puzzling feature has been the low degree of sequence variability in the counterpart of the HIV-1 V3 loop in HIV-2 and SIV_{mac} (1, 7, 66). The results in this paper suggest that the designation of V3 as a hypervariable region may have been unduly influenced by the characteristics of cultured isolates of HIV-1 and may not reflect the relative homogeneity of sequences in this region *in vivo*. The similarities between HIV-1 and other lentiviruses may be greater than were previously imagined.

In vivo phenotype of HIV. The sensitive and specific detection of actively replicating virus in cells by immunocytochemical staining for p24 antigen provided an opportunity to identify the main target cells of the sequence variants identified in this study *in vivo* and their associated cytopathology. The only infected cells that could be identified by this technique were tissue macrophages in the lung and either macrophages or microglial cells in the CNS. This macrophage infection was associated with tissue damage and with frequent giant cell formation.

In contrast, infected cells in the lymph node were not detected despite the detection of high frequencies of proviral sequences in DNA extracted from lymphoid tissue in both presymptomatic patients and those with AIDS. These findings, however, are consistent with previous reports of extensive but latent infection in lymphoid tissue (14), which were based upon the finding that the number of provirus-bearing cells within a lymph node greatly exceeded the number of those in which viral RNA sequences could be detected (58). Proviral loads calculated in this study and in others using similar techniques (58) range from 10^2 to 2×10^4 copies per 10^6 cells. These figures are not inconsistent with the reported high frequencies of provirus-bearing cells detected by *in situ* PCR (14, 15), as the former figures are for total lymph node DNA, which includes nucleic acids from cells not susceptible to HIV infection (B cells, follicular dendritic cells, cells within connective tissue, etc.).

The reason why HIV infection is largely latent in lymphoid tissue remains unclear. One possibility is that the DNA detected by PCR is partially reverse-transcribed provirus produced within extracellular virions (45, 75, 84). Alternatively, the DNA may be in the form of intracellular (cytoplasmic) partial transcripts previously observed *in vitro* upon exposure of HIV to cells that are nonpermissive for infection (21, 82, 83). As first-strand synthesis of provirus proceeds from the 3' end of the genomic RNA, transcripts for the 3' long terminal repeat and *env* should be relatively more abundant than transcripts of the *pol* and *gag* genes, as has been documented *in vitro* (82, 84). However, in this study careful quantitation by limiting-dilution PCR with nested primers showed no difference in the relative frequencies of V3 and *pol* region sequences, irrespective of whether the samples were from tissue of lymphoid or nonlymphoid origin (Tables 1 and 2; Fig. 1); this suggests strongly that the sequences detected and sequenced in this study originated from complete proviruses.

HIV tropism in vivo. We were unable to differentiate variants in this study in terms of SI/NSI phenotype, as all showed similar low charge and high degree of sequence conservation in the V3 loop. However, it was evident that differences existed in the populations of variants infecting

fluid ($\times 77$). (f) High-magnification ($\times 193$) view showing p24 antigen-expressing syncytia in the walls of alveoli. (g) Section of lung from patient 5 showing prominent lymphocytic infiltration (arrow) and containing carbon debris. The lung alveoli are partially filled with edema fluid ($\times 77$). (h) Distribution of p24 antigen within lung lymphoid tissue (arrow), similar to that observed in lymph nodes and spleen tissue.

different organs. For example, no patient showed equivalent distributions of variants in the CNS and in lymphoid tissue.

Although it is possible to document rapid turnover of *env* sequence variants with time in plasma of HIV-infected individuals (69, 79), little if anything is understood about the dynamics of sequence change outside the peripheral circulation. In particular, it is not known if separate populations of HIV develop in isolation from variants in other parts of the body (local evolution) or whether there is a process of continuous infection and spread from variants circulating in the blood (systemic evolution). Local evolution in nonlymphoid tissues might follow the widespread dissemination of HIV upon primary infection and persist at a low level (undetectable by PCR or by immunocytochemical staining) throughout the asymptomatic phase of infection because of cytotoxic T-cell activity or other immune effector mechanisms. This restriction on virus replication would become increasingly ineffective upon progression; the reactivation of virus replication in nonlymphoid tissue would form the basis for the previously observed redistribution of HIV in patients with AIDS (5, 13). In this model, different populations would develop in different tissues, through evolutionary drift and possibly through specific adaptive changes for replication in different cell types.

However, a consistent feature of the sequence distributions in this study and in others was the dispersed nature of many of the variants. For example, for many of the study patients, the major components of populations in brain and other nonlymphoid samples were often found as minor variants in lymphoid tissue (and vice versa), suggesting repeated traffic of virus between the two in patients who were severely immunosuppressed. These findings are more consistent with the hypothesis of systemic evolution of HIV, in which virus variants disseminate freely throughout different tissues in the later stages of infection and restrict the development of local populations.

One method of virus spread documented in this paper is by lymphocyte infiltration of a tissue. In patient 5, the high proviral load detected by quantitative PCR resulted from the formation of differentiated lymphoid tissue within the lung and was associated with the presence of a virus population which was indistinguishable from that in lymph nodes and spleen tissue. In contrast, widespread dissemination of infected macrophages most plausibly accounts for the sequence similarity between variants infecting lung, colon, and brain tissue in patient 4, since the sequence identity of the V3 loop would be unlikely to have arisen by chance at several sites in the body, as would be necessary in the local model of evolution.

What remains unclear is whether variants associated with infected macrophages are functionally distinct from those found in lymphoid tissue. We are currently investigating this question through further sequence comparisons of different parts of the *env* gene, and through in vitro characterization of isolates derived from lymphocyte- and macrophage-infiltrated tissues.

This study provides no information on the possible origins or fate of syncytium-inducing variants of HIV that are frequently isolated upon disease progression. In this study, not only did we fail to detect variants with a predicted SI phenotype in the patients with AIDS, but the observed behavior of HIV in vivo consistently differed from that observed with cultures. Immunocytochemical staining for p24 antigen in the CNS (and lung tissue) of the patients with AIDS revealed cytopathic infection of macrophage/microglial cells by variants with a predicted NSI phenotype. These findings suggest that in vivo observations of giant cell formation are not reproduced by infection of cell lines such as MT-2. Furthermore, the ability of HIV to infect

macrophages is not lost with disease progression as was previously suggested (10, 63). Indeed, the findings in this paper appear more consistent with previous findings that progression is accompanied by a change in the phenotype of isolates to SI variants that retain an ability to replicate in primary macrophage culture (8).

Independent evidence for the importance of macrophage tropism in pathogenesis has been obtained from observations of a more rapid CD4⁺ lymphocyte depletion in SCID-Hu mice infected with an MT variant than in those infected with SF-2 (53). In another animal model, the lack of disease progression, the stable CD4⁺ count, and the low circulating virus loads in chimpanzees infected with HIV-1 were attributed to a species-specific inability of HIV to infect chimpanzee macrophages (65). Directly or indirectly, the investigations described in this communication may contribute to our understanding of the mechanism by which T cells become depleted, the influence of macrophage infection, and the role of infection in the lymph nodes and spleen. These questions are essential for understanding the pathogenesis of HIV infection but are currently unresolved.

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