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Human immunodeficiency virus type 1 strains in the lungs of infected individuals evolve independently from those in peripheral blood and are highly conserved in the C-terminal region of the envelope V3 loop

(V3 domain/nucleotide divergence/monocyte trafficking/pulmonary alveolar macrophages)

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ABSTRACT **To determine whether human immunodeficiency virus type 1 (HIV-1) strains in the lungs of infected individuals are derived from proviral forms contemporaneously present in the peripheral blood or whether they evolve independently as an autonomous pool of viral quasispecies, HIV-1 envelope V3 domain structures at these sites were analyzed and compared. The V3 loop proviral nucleotide and inferred amino acid sequences from lung bronchoalveolar lavage, where HIV-1 is primarily found in macrophages, were more homogeneous within individuals than those from unseparated peripheral blood mononuclear cells, where virus is predominantly in T cells. Comparison between individuals revealed that strains from bronchoalveolar lavage, but not from peripheral blood mononuclear cells, contained V3 domain nucleotide sequences with a great degree of homogeneity in the C-terminal region and a highly conserved, negatively charged amino acid motif. This V3 loop C-terminal structure could be important in the ability of HIV-1 to infect alveolar macrophages. Phylogenetic analyses of V3 domain nucleotide sequences in cells of monocyte/macrophage lineage at both sites revealed the strains in lung macrophages to have evolved further from a presumed ancestral species than those in blood monocytes and to differ considerably in the inferred V3 loop amino acid structures. These results show that, as disease progression occurs, viral strains in monocyte/macrophage lineage cells within the lung and blood microenvironments are not in a state of unrestricted bidirectional traffic but, instead, evolve independently.**

At early stages of human immunodeficiency virus type 1 (HIV-1) infection, viral isolates from the peripheral blood of infected individuals generally grow slowly, do not induce syncytia (NSI), and are tropic for monocyte lineage cells (1). These strains are also found within macrophages at diverse tissue sites, including the lung (2), nervous system (3), and salivary glands (4, 5), where they may induce a variety of clinical manifestations. Progression of HIV-1 infection is associated with the appearance of syncytium-inducing (SI) isolates that replicate rapidly in T cells but not in macrophages (1, 6). However, even when the predominant isolate from the peripheral blood is T-cell tropic and SI, monocyte tropic and NSI HIV-1 strains continue to be isolated from extracirculatory sites such as the lung (6) and to be the predominant strains transmitted to a recipient (7, 8).

Tissue macrophages infected with HIV-1 are thought to be derived from monocyte lineage cells initially infected in the bone marrow or peripheral blood (9). Once infection becomes established, the mechanisms allowing for the persistence of viral infection in tissue macrophages remain unresolved. One possibility is that HIV-1 strains within circulating and tissue-based monocyte/macrophage lineage cells are in continuous and unrestricted communication and that viral strains in peripheral tissues reflect proviral forms that are contemporaneously present in the blood (10). This mechanism, however, does not account for the differences that are sometimes observed in the biological phenotypes of strains simultaneously isolated from blood and tissues. An alternative possibility is that after the initial infection of tissue macrophages, a self-perpetuating cycle develops, with locally elaborated virus infecting both uninfected resident macrophages and new waves of freshly recruited monocyte lineage cells. Within the tissue microenvironment, HIV-1 evolution would then be under the influence of local factors such as cytokines elaborated during inflammatory conditions (2), resident copathogens capable of viral transactivation (11), or compartmentalized antiviral host immune responses (5, 12).

HIV-1 tropism for macrophages or T cells correlates, in part, with structural differences within the HIV-1 gp120 envelope, most notably with the presence of particular residues in a 35-amino acid region within the highly variable principal neutralizing V3 domain (13–15). The gp120 V3 loop is the target of both neutralizing antibodies (16, 17) and cytotoxic T cells (18) elicited by natural infection, and the high rates of sequence change in this region are thought to confer adaptive value to HIV-1 by allowing it to evade these host immune responses (19–21). In the present study, we sought to determine whether HIV-1 strains within the blood and lungs of the same infected individuals differed in the structures of the V3 domains. To test the possibility that HIV-1 strains within the same cell lineage evolve differently in the lung environment than those in blood, phylogenetic studies were performed to determine the relationship between V3 domain sequences from circulating monocytes and tissue macrophages. Our results support the concept that persistence of HIV-1 strains within an extracirculatory microenvironment reflects independent evolution and autonomous cycles of local viral replication.

METHODS

Determination of HIV-1 V3 Domain Sequence in Bronchoalveolar Lavage (BAL) and Blood. HIV-1 V3 loop proviral DNA sequences were determined by polymerase chain reaction (PCR) amplification of DNA obtained simultaneously from unseparated mononuclear cells in BAL and peripheral blood of three individuals infected with HIV-1, as described

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage.

(22). In two of these individuals, V3 loop sequences were also determined in the adherent fraction of the circulating mononuclear cells and these were compared to sequences predominating in the BAL. In a fourth individual, BAL sequences alone were obtained. Patients with symptomatic pulmonary disease (bacterial pneumonia, *Pneumocystis carinii* pneumonia, *Mycobacterium avium* intracellulare complex, Kaposi sarcoma) were intentionally selected to maximize the chance of observing divergent viral evolution in the blood and lungs. For each person, 15–20 clones from both BAL and blood were sequenced using an automated DNA sequencer (Applied Biosystems).

Sequence Analyses and Tree Alignment. For each individual studied, V3 loop nucleotide sequences of all quasispecies were aligned and the majority nucleotide consensus sequences were obtained. Evolutionary distances between nucleotide sequences of quasispecies present in each individual were determined using the two-parameter model of Kimura for pairwise comparisons (23). Pairwise comparisons of sequences from the BAL or peripheral blood were pooled for all patients, enabling mean intra- and interpatient nucleotide divergence at each site to be calculated. Results are expressed as mean nucleotide divergence ± standard deviation. Statistical significance for differences between means and homogeneity of distribution about the mean were calculated using Levene's test for equality of variances. Phylogenetic trees were generated by using the PHYLIP package provided by J. Felstenstein (24). SEQBOOT was used to generate 100 unique sets of sequences from the input of aligned sequences. These sets were then entered into the maximum DNA parsimony program DNAPARS and a consensus tree was generated using CONSENSE. Phylogenetic trees were also generated by the distance matrix method of Fitch and Margoliash (25) by using the programs DNADIST and FITCH. This enabled calculation of branch lengths from the presumed ancestral species, identified by both phylogenetic algorithms, to each contemporary sequence.

HIV-1 Proviral DNA Quantitation in Adherent and Nonadherent BAL Cell Fractions. DNA was extracted from 1×10^6 uncultured BAL mononuclear cells that were separated by plastic adherence for 1 hr. The proportions of monocytes and lymphocytes in the adherent and nonadherent fractions were determined by staining the permeabilized fractions with CD68 and CD3 monoclonal antibodies. HIV-1 gag was then amplified from samples of each DNA fraction by PCR using 5′-CAAGCAGCCATGCAAATGTTAAAAGA-3′ and 5′-ATTTTGGACCAACAAGGTTTCTGTC-3′ as primers. The amplified product was hybridized to a ^{32}P-labeled oligonucleotide probe and total copy numbers were quantified by a Betagen Betascope using 8E5 cells as standard. The results were expressed on a per cell basis by normalizing to amplified conserved regions of HLA-DQa.

RESULTS

Comparison of HIV-1 V3 Domain Proviral DNA Sequences and Inferred Amino Acid Structures from BAL and Unseparated Peripheral Blood Mononuclear Cells (PBMC) Within the Same Individuals. The intrapatient nucleotide divergence between sequences in BAL ranged from 2.2% to 3.7% (mean, 2.7% ± 2.6%) and between those in unseparated PBMC from 0.8% to 6.9% (mean, 4.9% ± 4.1%). The difference between the means of the two groups, 2.2%, was significant, $P < 0.0001$. After grouping together the proviral sequences obtained from the BAL and unseparated PBMC of the same individual, the intrapatient nucleotide divergence between sequences from both sites was found to range from 2.5% to 6.8% (mean, 5.1% ± 4.1%). In each individual, the intrapatient nucleotide diversity between sequences from BAL and blood was greater than the diversity between sequences within either site alone, indicating that the strains at these two sites were highly divergent.

The inferred V3 loop amino acid sequences of quasispecies present in BAL and peripheral blood of two individuals are presented in Fig. 1. Aligning all V3 loop proviral sequences to the majority consensus from BAL, no sequence was shared between the blood and BAL of these patients. In patient 3, not shown, there were more similarities, with 3/8 sequences from blood being identical to 7/10 sequences from BAL. In patient 1, all 10 BAL sequences contained lysine, valine, and aspartic acid residues at V3 loop positions 18, 26, and 29, respectively. In contrast, 5/10 sequences from the blood of this patient had lysine → serine/glutamic acid at residue 18, 10/10 had valine → isoleucine at residue 26, and 5/10 had aspartic acid → asparagine at residue 29. In addition, leucine was found at position 4 in 8/10 BAL sequences, while proline was found at this position in 6/10 sequences from blood. In patient 2, all 8 BAL sequences contained arginine, tyrosine, threonine, and aspartic acid at V3 loop positions 10, 21, 23, and 29, respectively. In contrast, all 8 sequences from blood had arginine → serine, tyrosine → isoleucine, threonine → arginine, and aspartic acid → asparagine substitutions. Of the four most divergent amino acid residues between BAL and blood in each individual, only aspartic acid at position 29 was shared by every BAL sequence. All sequences from the BAL of patient 3 also contained a negatively charged residue, aspartic or glutamic acid, at this position (data not shown).

Comparison of HIV-1 V3 Domain Proviral DNA Sequences and Inferred Amino Acid Structures from BAL and PBMC of

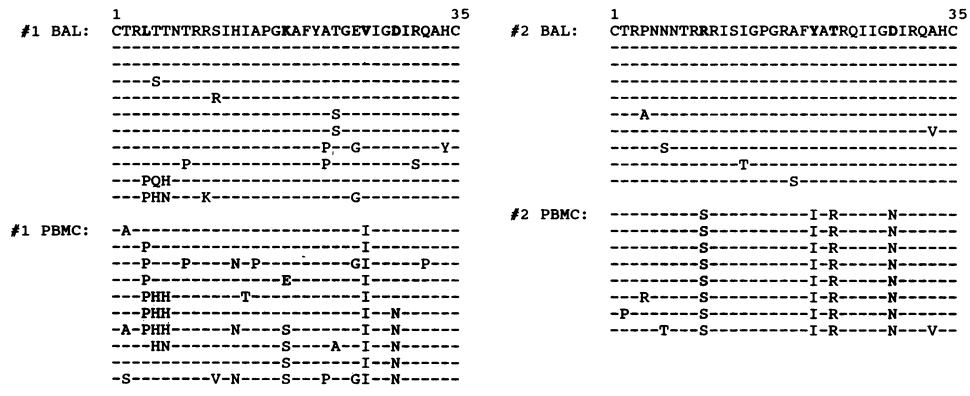

FIG. 1. Inferred V3 loop amino acid sequences of HIV-1 quasispecies present in BAL and peripheral blood of two individuals. Aligning all sequences from the blood to the consensus sequence from the BAL of each particular individual demonstrates that, within the same host, strains present at these two sites differ greatly. Amino acids in the BAL consensus that differ in 50% of sequences from the blood are shown in boldface type.

Unrelated Individuals. The mean interpatient sequence diversity of the V3 domain was found to be narrower in BAL (351 comparisons) than in PBMC (276 comparisons) (11.0% ± 6.4% vs. 14.5% ± 8.2%, $P < 0.0001$). To examine whether these differences were present throughout the V3 domain, or were limited to a particular region, interpatient comparisons were repeated for sequences spanning the regions at either the N-terminal or C-terminal sides of nucleotides encoding the conserved crown of the V3 loop (26). As shown in Fig. 2, the distribution of interpatient nucleotide distances at the C-terminal region of the V3 domain was significantly narrower in BAL (Fig. 2*C*), with closer clustering about the mean, than the distribution in PBMC (Fig. 2*D*) (6.6% ± 3.8% vs. 10.0% ± 7.1%, $P < 0.0001$). In contrast, the distribution of interpatient nucleotide distances and the mean interpatient divergence at the N-terminal region of the V3 domain did not differ significantly between BAL (Fig. 2*A*) and PBMC (Fig. 2*B*) (16.1% ± 10.4% vs. 18.5% ± 10.5%, $P = 0.27$).

As shown in Fig. 3, all three consensus sequences from the BAL contained tyrosine at V3 loop residue 21, threonine at residue 23, and aspartic acid at residue 29, in common with a prototypic macrophage tropic HIV-1 strain ADA. Thirty-five of 38 V3 loop amino acid sequences (92%) obtained from the BAL of these three plus a fourth individual contained the motif Tyr-Xaa-Thr-Xaa-Xaa-Xaa-Ile-Gly-Asp from residues 21 to 29 on the C-terminal side of the V3 crown. In contrast, this motif was only present in 13/33 sequences (39%) from the peripheral blood of these persons ($P < 0.001$). As shown in Fig. 4, this motif is also present in the V3 loops of other laboratory strains that are able to infect macrophages, but it is not present in strains HXB2 and SF2 that cannot.

Cellular Localization of HIV-1 DNA in BAL. Quantitative HIV-1 proviral DNA studies were performed on adherent and nonadherent cellular fractions from the BAL of patients 1 and 2. In each patient, >90% of the BAL adherent cells were of macrophage lineage and contained from 3000 to 5000 copies of viral *gag* DNA per 10^6 cells. In contrast, the nonadherent cellular fractions contained 80- to 100-fold less viral *gag* DNA, demonstrating that the vast majority of proviral DNA in the BAL of these patients was in the lung macrophages.

```
                                1                                   35
HIV-1 ADA (M-tropic)    CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC

Patient   #3 BAL        ------------------------D----------
          #3 PBMC       ------------------------D----------

Patient   #2 BAL        ---------RR-S--------A-RQ----------
          #2 PBMC       ---------SR-S-------IARRQ---N------

Patient   #1 BAL        ---LTT---R----A--K---A---V---------
          #1 PBMC       ----TT---R----A--K---A------N------
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FIG. 3. Unrelated strains from BAL of different individuals have a distinctive amino acid motif in the V3 loop C-terminal region. In common with a prototypic macrophage tropic strain, HIV-1 ADA, all three consensus sequences from BAL contained tyrosine at V3 loop residue 21, threonine at residue 23, and aspartic acid at residue 29 (boldface type). In contrast, two of three consensus sequences from the peripheral blood of these patients had substitutions at one or more of these residues (boldface type). The motif Tyr-Xaa-Thr-Xaa-Xaa-Xaa-Ile-Gly-Asp from residues 21 to 29 on the C-terminal side of the HIV-1 V3 crown was present in all three BAL sequences but in only one of three sequences from the blood.

Structure and Phylogenetic Relationship of V3 Domains from HIV-1 Strains Present in Monocyte/Macrophage Lineage Cells in BAL and PBMC of The Same Individuals. Because HIV-1 V3 loop structures in unseparated blood are more likely than those in BAL to contain contributions from T cells, HIV-1 V3 domain nucleotide sequences from cells limited to monocyte/macrophage lineage were compared between BAL and peripheral blood of patients 1 and 2. In contrast to the situation with unseparated PBMC, the peripheral blood adherent cell fraction, enriched for >70% monocyte lineage cells as determined by staining with CD14 and CD68 monoclonal antibodies, had HIV-1 V3 domain

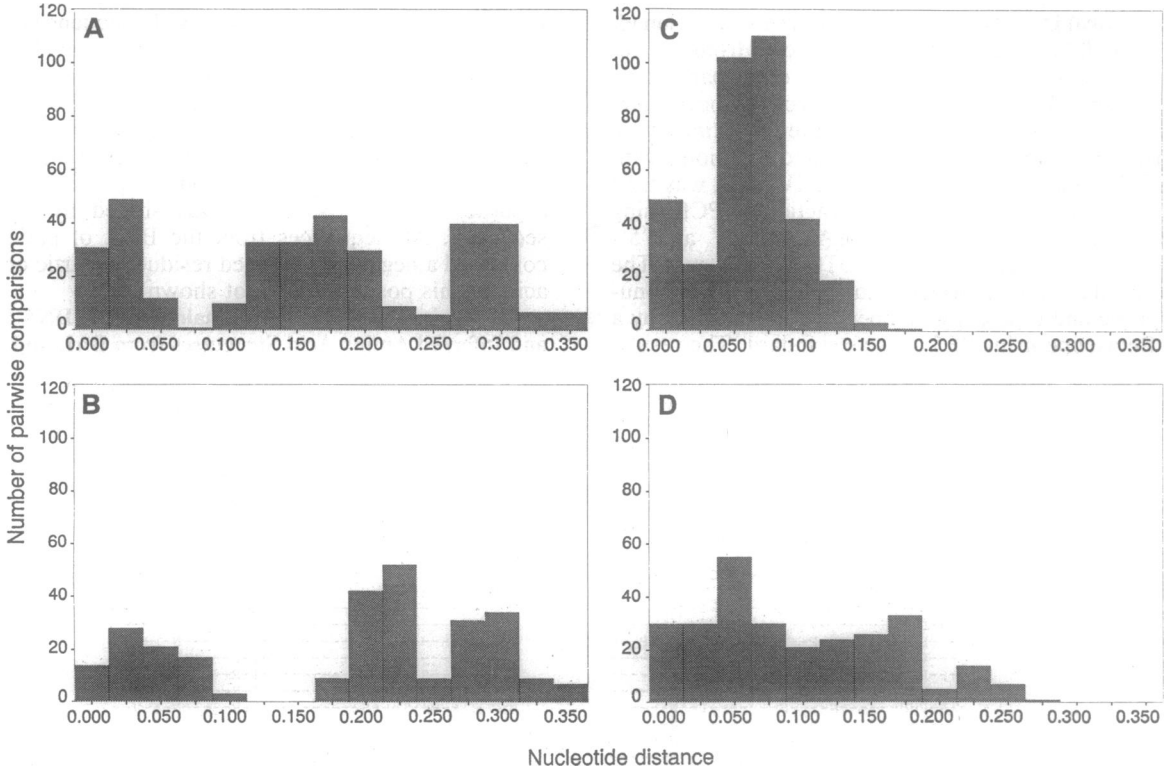

FIG. 2. Distributions of interpatient nucleotide distances between sequences spanning the regions at either the N-terminal or C-terminal sides of nucleotides encoding the conserved crown of the HIV-1 V3 loop. The distribution of interpatient nucleotide distances at the C-terminal region of the V3 domain was significantly narrower in BAL (*C*) than the distribution in PBMC (*D*). The distributions at the N-terminal region did not differ significantly between BAL (*A*) and PBMC (*B*).

Macrophage infection	HIV-1 Strain	1 CTRPNNNTRKSIHI..GPGRAFYTTGEIIGDIRQAHC 35
+	ADA	----------
+	JR-FL	----------
+	Bal	----------L----------
+	SF162	----------T----------A--D----------
+	W2 1b4-S	---------R--TM------VL----A----------
-	HXB2	----------R-R-QR------V-I-K.--MM-----
-	SF2	----------Y---------H---R------K---

FIG. 4. The motif Tyr-Xaa-Thr-Xaa-Xaa-Xaa-Ile-Gly-Asp from residues 21 to 29 on the C-terminal side of the HIV-1 V3 crown is found in the V3 loop of laboratory strains that are able to infect macrophages but is not present in strains that cannot.

		1 35
Patient	#1 PBMC	CTRPTTNTRRSIHIAPGKAFYATGEIIGNIRQAHC
	#1 Monos	----NN---K----------D------
	#1 BAL	---L----------V--D------
Patient	#2 PBMC	CTRPNNNTRSRISIGPGRAFIARRQIIGNIRQAHC
	#2 Monos	---------G----------
	#2 BAL	---------R----------Y-T-----D------

FIG. 5. Comparisons between consensus HIV-1 V3 loop amino acid sequences derived from blood monocytes (Monos) with those from BAL macrophages of patients 1 and 2. Sequences are aligned with the consensus from unseparated PBMC. In each individual, the V3 loop structures in blood monocytes differed from those in BAL at both the N-terminal and C-terminal regions on either side of the V3 loop crown (boldface type).

nucleotide sequences that were less divergent than those in BAL (mean of pooled intrapatient pairwise comparisons, 1.2% ± 1.4% vs. 3.1% ± 2.9%, $P < 0.0001$). Fig. 5 shows the V3 loop consensus amino acid sequences from the blood adherent cells of patients 1 and 2, demonstrating that the V3 loop structures of strains in these cells differed from both those in BAL and those in unseparated PBMC. In each patient, 9/10 sequences from the blood adherent cells contained all of the amino acid substitutions defined by their consensus sequences. In patient 1, the V3 consensus sequence from the blood adherent cells differed from the BAL consensus sequence by four residues in the N-terminal half of the V3 loop and by one residue in the C-terminal half. In patient 2, the V3 loop consensus sequence from the blood adherent cells differed from the BAL consensus sequence by one residue in the N-terminal half and by three residues in the C-terminal half.

To establish the evolutionary relationships between the strains present in the monocyte-enriched fractions of peripheral blood and BAL, phylogenetic analyses were performed using both parsimony and distance matrix algorithms (24). By using the DNAPARS and CONSENSE programs, a branching node could be identified in each individual as separating all BAL sequences from all those in the blood adherent cells. Bootstrap analysis of 100 replicates showed that such a branch node was present in 93 and 94 of 100 trees (95% confidence interval) in each of the studied patients. The same branch nodes were identified by the phylogenetic trees constructed using the Fitch–Margoliash distance matrix procedure (25) (Fig. 6). If one assumes that the BAL and blood adherent cell proviral sequences have evolved from a common ancestor, the mean evolutionary divergence of sequences at each site from the presumed ancestral node could be calculated by measuring the branch lengths from this node to each sequence. In both individuals studied, the mean nucleotide divergence from the presumed ancestral species was significantly greater for proviral strains in the BAL than for strains in the blood adherent cells (6.0% vs. 2.7%, $P = 0.0002$, and 5.2% vs. 1.4%, $P = 0.0001$).

DISCUSSION

There are two central observations in this report. The first is that, within a single individual, HIV-1 strains tropic for cells of the same lineage but residing in different microenvironments are not in a state of unrestricted bidirectional traffic but, instead, evolve independently. Second, comparison of the V3 domain structure of HIV-1 strains present in BAL and peripheral blood of unrelated individuals revealed the C-terminal region of strains in BAL to have a narrow nucleotide divergence and a relatively conserved amino acid sequence. Strains in BAL of the four patients studied had a markedly increased frequency of the motif Tyr-Xaa-Thr-Xaa-Xaa-Xaa-Ile-Gly-Asp in the V3 loop C-terminal region compared with those in whole blood. Because HIV-1 DNA in BAL was found almost exclusively in the macrophage-enriched fraction, these findings raise the possibility that this V3 loop structure may be important in the ability of HIV-1 to infect alveolar macrophages.

Alveolar macrophages are the principal cell type infected by HIV-1 in the lung, as demonstrated by immunohistochemical techniques (12) and cellular localization of viral RNA and DNA (10, 27, 28). These cells are much more susceptible to HIV-1 infection than are circulating monocytes and produce far higher levels of infectious virus after primary inoculation with macrophage tropic viral isolates (29). Once infection becomes established, the mechanisms allowing for the persistence of viral infection in tissue macrophages remain unresolved. Proponents of the "Trojan horse hypothesis" (10) have suggested that HIV-1 strains within monocyte/macrophage lineage cells in tissues are derived from those contemporaneously present in circulating monocytes and that new provirus in a latent form from the blood constantly enters peripheral tissues. Against this model, however, is the observation that strains isolated from BAL of individuals at various stages of disease are uniformly macrophage tropic and maintain this biologic phenotype even when the predominant isolates from the blood of the same persons are T-cell tropic (6).

Our demonstration of marked intrapatient divergence between V3 loop structures in the blood and lungs supports the alternative concept that the viral populations at these two sites evolve independently (2, 6) and enable us to propose the following model. Following initial infection with HIV-1, monocyte lineage cells derived from the bone marrow or peripheral blood seed the lungs and other tissues where they

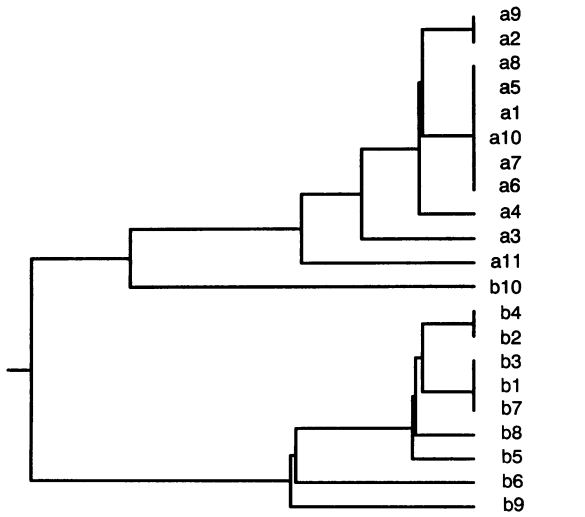

FIG. 6. Phylogenetic tree, constructed using the Fitch–Margoliash algorithm, analyzing the evolutionary relationship between HIV-1 V3 domain sequences in monocyte/macrophage lineage cells from the blood (a) and lung (b) of a representative individual (patient 1). This analysis demonstrates two almost completely separate phylogenetic clusters, indicating a high degree of divergence between strains at these sites.

differentiate into macrophages (9). Here, the virus establishes a relatively autonomous cycle of replication that is dependent on local cofactors, including cytokines (2) and viruses (11) capable of transactivating HIV-1, and on a renewable source of uninfected monocyte lineage cells trafficking from the blood. In addition, viral strain evolution at these sites may be influenced by local anti-HIV host immune responses, which can develop prominently within macrophage-rich tissues such as the lungs (12) or the salivary glands (5, 30) of certain individuals.

The observation that unrelated HIV-1 strains within alveolar macrophages of different individuals had a high degree of homogeneity in the nucleotide sequences encoding the C-terminal region of the V3 domain, but not those encoding the N-terminal region, suggests that the former are under evolutionary selection pressures preventing their divergence. Examination of the V3 loop amino acid sequences from BAL supported this conclusion, with a conserved motif at the C-terminal end being enriched at this site. A conserved V3 motif has also been reported in strains from brain tissues compared to those in blood (26), raising the possibility that viral tropism for diverse tissues is dependent on distinct V3 loop structures. In contrast, the wide interpatient nucleotide and amino acid divergence at the N-terminal end of the V3 domain suggests that this region could be evolving under immunological selection pressures that differ from individual to individual and depend on HLA type and T-cell receptor repertoire (22).

Evidence has previously been presented that patterns of HIV-1 cellular tropism either for monocytes/macrophages or for T cells correlate with structural differences within the gp120 envelope, most notably the presence of particular charged amino acids within the V3 domain (13-15). Since HIV-1 in the lung is primarily localized to alveolar macrophages, the V3 loop C-terminal motif identified in this report, which encompasses negatively charged residues previously associated with macrophage tropism *in vitro* (15), may encode a structure that is critical for a particular aspect of the viral life cycle within the lung microenvironment, such as attachment to or fusion with the cell membrane, or intracellular processing of viral proteins. This possibility is supported by the observation that the motif is also present in laboratory strains that are able to infect macrophages, such as HIV-1 ADA, but not in those that are unable, such as HXB2 and SF2. Further experiments must be undertaken to determine whether this motif influences tropism for cells of monocyte/macrophage lineage.

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