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Limited sequence heterogeneity among biologically distinct human immunodeficiency virus type 1 isolates from individuals involved in a clustered infectious outbreak

(monocyte/tropism/lentivirus/divergence/envelope)

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ABSTRACT **Human immunodeficiency virus type 1 isolates were obtained over a 3-year period from blood, brain, and lung of three patients in a clustered infectious outbreak. This included a blood donor who was initially asymptomatic but subsequently developed AIDS-related complex and two neonatal transfusion recipients who developed AIDS. Isolates from brain and lung replicated to >30-fold higher levels in primary monocyte cultures than did those from blood; no growth differences on primary lymphocytes were observed. Thirteen clones were obtained from seven isolates, and *env* sequences were determined. The predicted amino acid sequences among these clones differed by only 0.01% but differed by 15–27% when compared to previously sequenced isolates from other patients. The level of envelope amino acid sequence divergence noted among these isolates is considerably lower than that previously reported for other human immunodeficiency virus isolates. No differences in the envelope unique to lung or brain isolates compared to blood isolates were noted. This study provides evidence that mutations in the envelope may not be necessary for disease progression and that other portions of the viral genome may contribute to cell-specific tropism.**

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subgroup of retroviruses, which includes visna virus, equine infectious anemia virus, and caprine arthritis encephalitis virus (1). These viruses cause relapsing or slowly progressive diseases associated with the emergence of antigenically distinct viral variants (2). In addition, they are tropic for monocytes, a potential sanctuary from immune surveillance mechanisms (3–6).

Individuals infected with HIV-1 may remain asymptomatic for many years, but at least half ultimately develop progressive generalized lymphadenopathy, constitutional symptoms termed AIDS-related complex (ARC), or AIDS (7). Factors that may influence disease progression include host- and virus-specific determinants (8).

HIV-1 isolates display sequence heterogeneity between strains (9, 10). The area of greatest diversity is in the *env* gene, especially the portion encoding the surface protein (SU; gp120) (10). Within *env*, there are conserved and variable sequences (10).

The variation in envelope amino acid sequences is 3–27% among isolates from different individuals (10). Sequence differences of 1–3% have been noted between related virus isolates obtained from an infected child or an infected laboratory worker (11, 12). Mechanisms to account for this heterogeneity may include infidelity of the reverse transcriptase, copy-choice switches, recombination, and selective pressure of the host's immunosurveillance (13).

The *env* gene of HIV-1 encodes a glycoprotein of 160 kDa that is cleaved to SU, a 120-kDa extracellular glycoprotein, and a 41-kDa transmembrane protein (TM) (9). The envelope glycoprotein is responsible for attachment to T lymphocytes by way of the CD4 antigen (14). This is critical for virus-cell fusion for infectivity and for cell–cell fusion for cytopathicity (15, 16). The envelope is also the most immunogenic protein of HIV-1 (17, 18) and has been characterized in detail to facilitate vaccine development.

In this study, we have determined the degree of *env* heterogeneity of HIV-1 isolates derived from a clustered outbreak and have found a much lower level of sequence heterogeneity than previously described (10–12), despite remarkable differences in biological properties.

MATERIALS AND METHODS

Virus Isolation. Virus isolation was performed by coculturing peripheral blood mononuclear cells (PBMCs) from patients with an equivalent number of phytohemagglutinin-activated PBMCs from an uninfected donor (19). Cultures were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, interleukin 2 (20 units/ml), 4 mM glutamine, 50 μM 2-mercaptoethanol, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cultures were split to a density of one million cells per ml every 3–4 days. An equal number of fresh phytohemagglutinin-activated PBMCs from an uninfected donor were added weekly for 3–6 weeks.

For cultures from brain or lung tissue, samples were homogenized on ice in a Virtis homogenizer and filtered through a 0.2-μm filter (20). The filtrate was added to PBMCs of uninfected donors and cultured as described above.

Cultures were monitored by reverse transcriptase measurements (21), and cells were harvested at the time of peak reverse transcriptase production, at approximately 21 days. Cultures were maintained by five investigators working with two incubators and biosafety cabinets in different buildings and at different times to avoid cross-contamination.

Monocyte Cultures. Cultures of primary human monocytes were prepared from PBMCs by Ficoll and allowed to adhere for 5 days to plastic culture flasks containing RPMI 1640 medium supplemented with 20% fetal calf serum, 10% (vol/vol) HIV-1-seronegative human serum, 4 mM glutamine, 50 μM 2-mercaptoethanol, penicillin (50 units/ml), and strepto-

Abbreviations: ARC, AIDS-related complex; HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PGL, progressive generalized lymphadenopathy; SU, surface protein; TM, transmembrane protein.

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mycin (50 μg/ml). The cells were then treated with monoclonal antibody OKT3 and complement to obtain cultures >95% pure monocytes (22). Monocytes were subsequently cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM glutamine, 50 μM 2-mercaptoethanol, penicillin (50 units/ml), and streptomycin (50 μg/ml). To some cultures, granulocyte–macrophage colony-stimulating factor (Genzyme) at 2 units/ml, macrophage colony-stimulating factor at 15 units/ml, or recombinant human interleukin 3 (Genzyme) at 5 units/ml were added. Cultures were monitored by reverse transcriptase measurements (21) or soluble p24 antigen measurements (DuPont).

Molecular Cloning. Total cellular DNA or Hirt DNA was isolated from positive cultures as described (23, 24). Cellular DNA was digested to completion with *Sac* I and ligated to *Sac* I λZAP arms (Stratagene). Libraries were screened with a 3′ hexamer labeled probe derived from the 3.6-kilobase *Sac* I fragment of clone pHXB2 (nucleotides 5580–9154) (25, 26). Positive plaques were purified and excised as plasmid according to manufacturer's specifications. Clones were isolated independently by three investigators working at different times in the laboratory.

Polymerase Chain Reaction (PCR) Amplification. One-microgram DNA samples from uncultured patient tissue were subjected to PCR amplification (27). Reaction mixtures were as described by the manufacturer (Cetus) utilizing 1 unit of *Thermus aquaticus* polymerase, primers corresponding to nucleotides 1810–1828 and 2241–2265, and a Perkin–Elmer/Cetus thermocycler for 40 cycles of 2.5 min at 94°C, 2.0 min at 53°C, and 10 min at 72°C. PCR products were purified by agarose gel electrophoresis and DEAE-cellulose chromatography (23).

Nucleotide Sequence Analysis. Sequencing was performed by a modification of the dideoxynucleotide method (28) using either Sequenase or Sequenase 2.0 T7 polymerase according to manufacturer's recommendations (Stratagene), with a set of oligonucleotide primers along the length of the HIV-1 *env* gene.

RESULTS

HIV-1 Isolates. Isolates were obtained from three patients involved in a clustered outbreak (Fig. 1). Patient I was an asymptomatic infected homosexual at the time he donated blood in February 1985. An isolate was obtained from his blood in August 1986 while he was asymptomatic and in June 1988 after he developed ARC. Patients II and III were recipients of the unit of blood donated by patient I, shortly after their premature births. An isolate was obtained from the blood of patient II in February 1986 after patient II developed progressive generalized lymphadenopathy, from blood in August 1986 after patient II developed AIDS, and from brain and lung tissues obtained at autopsy in September 1986. Isolates were obtained from the blood of patient III after he had developed AIDS in February 1986, November 1986, April 1987, and June 1987 and from brain tissue obtained at autopsy in June 1987.

Cell-Specific Tropism. Growth characteristics of these isolates were assessed on primary lymphocytes and monocytes. No differences in their replicative capacities were noted on lymphocytes (data not shown). However, on monocytes, the brain and lung isolates generated >30-fold and >50-fold higher levels of virus, respectively, than the isolates from blood (Fig. 2). Virus derived from the molecular clone HXB2 was unable to replicate on primary monocytes in this experiment (data not shown). Similar results were obtained in the presence of macrophage colony-stimulating factor or interleukin 3 or in the absence of specific growth factors (data not shown).

***env* Sequence Difference.** Clones of the 3′ portion of the viral genome were obtained from seven HIV-1 isolates (Fig. 1) and the *env* gene of each clone was sequenced. Primary sequence data are available through GenBank (M31451). Table 1 and Fig. 3 compare the sequences from clone I-BL2 with those of 14 other HIV-1 sequences (10). Clone I-BL2 was obtained from patient I at a time when he was asymptomatic and has been chosen as the reference clone to which all comparisons are made. The predicted amino acid sequences of clone I-BL2 differ from those of clone HXB2 at 15% of positions. The sequence differences have been analyzed separately for variable and constant domains and demonstrate a similar pattern of diversity seen in previous investigations (10, 29). Amino acid sequence differences in clone I-BL2 compared to clone HXB2 envelope of 38–70% are noted in the variable domains and 4–11% in the conserved domains (Table 1).

The CD4 binding domain within the C3 region (30) of clone I-BL2 demonstrates no distinctive differences compared to those of other clones previously analyzed (10). Seventeen

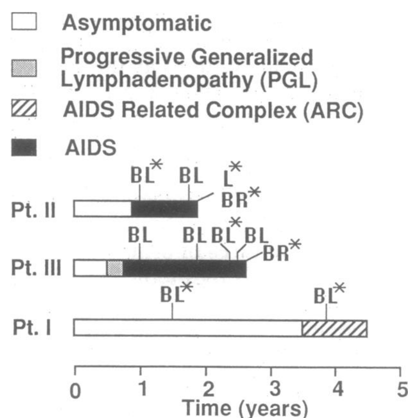

FIG. 1. Sources of HIV-1 isolates. The clinical course of each patient is summarized as well as the time of isolation of HIV-1 from blood (BL), brain (BR), or lung (L). Patient I was an asymptomatic HIV-1-positive homosexual whose blood was transfused into patients II and III shortly after their premature births. Patient (Pt.) I developed ARC 40 months later. Patients II and III developed AIDS at ages 8 and 7 months, respectively. Asterisks indicate isolates from which clones were derived.

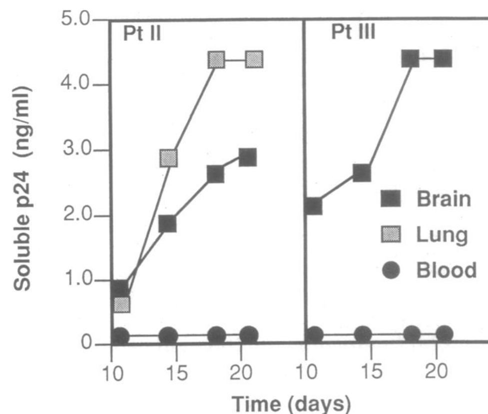

FIG. 2. Enhanced replication on primary human monocytes of HIV-1 isolates from brain and lung tissue compared to those from blood. Equivalent amounts of each isolate obtained from patient (Pt) II or III were grown on primary human monocytes in the presence of granulocyte–macrophage colony-stimulating factor (2 units/ml). Three blood isolates from patient III gave indistinguishable results and are represented by a single line. Soluble p24 antigen measurements were used to monitor virus production. All four isolates replicated at an equivalent level on primary human lymphocytes.

Table 1. Comparison of clone I-BL2 amino acid sequences with published envelope sequences

Region	Positions	Range of variation, %	Differences, %
V1	129–148	36.0–71.4	70.0
V2	157–192	2.8–77.8	38.9
V3	297–325	3.4–68.9	37.9
V4	388–411	0.0–82.6	70.8
V5	455–466	23.0–50.0	50.0
C1	29–128	1.0–16.0	4.0
C2	197–271	0.0–13.7	10.7
C3	414–454	4.9–19.5	7.3
C4	468–505	0.0–15.8	7.9
C5	506–613	0.9–12.0	4.6
C6	647–739	2.2–20.4	9.7
Total	1–851	3.3–27.0	15.0

Positions of constant (C) and variable (V) domains are based on the work of Modrow *et al.* (29). Range of variation is for clone HXB2 versus previously published sequences (10). Differences between clones HXB2 and I-BL2 are shown.

cysteine residues in envelope are conserved with previously reported sequences. An additional cysteine residue is found in the signal peptide at residue 10, and the cysteine at residue 831 in 4 of 14 HIV-1 clones is not present in clone I-BL2. Twenty-two of 24 conserved potential N-glycosylation sites are found in clone I-BL2, but three additional sites in I-BL2 envelope are not found in most previously sequenced HIV-1 envelopes.

Sequence differences among the 13 clones obtained from seven isolates of the three patents involved in the clustered outbreak are shown in Table 2. Seven nucleotide differences are noted compared to clone I-BL2 at four positions, and one predicted amino acid difference is present. Thus, the level of nucleotide sequence divergence among these clones averages 0.02%, and the amino acid sequence divergence averages 0.01%. No novel sequence differences are noted in clones derived from isolates that demonstrate high replicative capacity in monocytes (asterisks in Table 2).

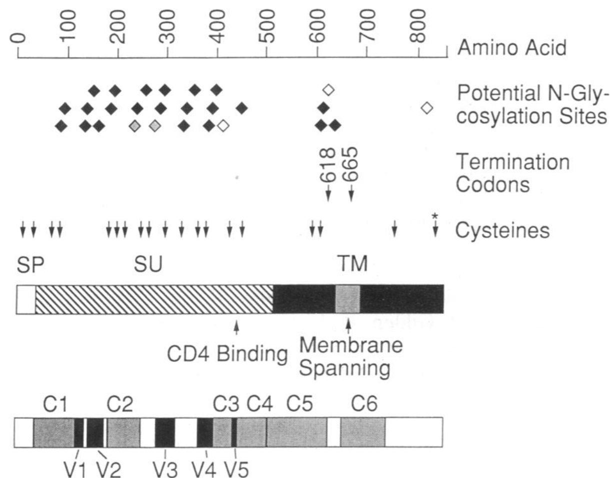

FIG. 3. Comparison of envelope sequence from clustered outbreak with envelope sequences previously reported. Positions of conserved potential N-glycosylation sites present in 85% of reported clones (10) and in clone I-BL2 (◆), conserved potential N-glycosylation missing from clone I-BL2 (◈), and new potential N-glycosylation sites present in clone I-B2 (◇) are shown as well as the major structural regions of the envelope protein, signal peptide (SP), SU, TM, including the membrane-spanning domain (30). The positions of termination codons found in each clone derived from the clustered outbreak and the relative positions of variable (V) and constant (C) domains of envelope (10) are shown.

Table 2. Sequence diversity in *env* among HIV-1 clones from individuals infected with a common progenitor virus

Clone	Nucleotide				Amino acid
	168	1583	1584	2184	528
I-BL2	T	C	G	C	Ala
I-BL10	—	—	—	—	—
I-BL1	—	—	—	—	—
I-BL9	C	—	—	—	—
II-BL1	—	—	—	—	—
II-BL6	—	—	—	—	—
II-L17*	C	G	C	—	Arg
II-BR1*	—	—	—	—	—
II-BR3*	—	—	—	—	—
III-BL1	—	—	—	—	—
III-BL6	—	—	—	A	—
III-BR2*	C	—	—	—	—
III-BR3*	C	—	—	—	—

Roman numerals indicate patient from whom clone was isolated; letters indicate tissue source of HIV-1 isolate (BL, blood; L, lung; BR, brain). Clones I-BL2 and I-BL10 are derived from patient I while asymptomatic and clones I-BL1 and I-BL9 were derived from the patient when symptomatic. Dashes indicate identical nucleotide or amino acid compared to that present in clone I-BL2.
*Clones derived from isolates that replicated well on monocytes.

Six hundred nucleotides determined directly from PCR products from two fresh tissue samples of these patients demonstrate no nucleotide differences from clones derived from viral isolates of these same tissues (data not shown). Identical termination codons were demonstrated in fresh tissue DNA amplified by PCR.

In addition, all 13 clones demonstrated two termination codons at positions 618 and 665, positioned on the N-terminal side of the membrane-spanning domain and within the beginning of this domain of TM, respectively (Fig. 3).

DISCUSSION

The current study represents a detailed molecular analysis of isolates obtained from individuals involved in a clustered HIV-1 infectious outbreak. These isolates were obtained from individuals whose clinical courses represent a wide range of manifestations of HIV-1 infection, including isolates obtained from an adult at times when he was either asymptomatic or had ARC and from three different tissues of neonatally infected children.

Cell-Specific Tropism of HIV-1 Isolates. This study confirms previous investigations demonstrating cell-specific tropism (31, 32). Isolates derived from brain and lung could infect monocytes and replicate to significantly higher levels than isolates derived from blood. Monocytes are the predominant infected cells in the brain and lung (33, 34), whereas lymphocytes may be the predominant host cells in blood (35). However, monocyte-tropic isolates have also been obtained from blood, suggesting that peripheral blood monocytes are also a suitable host for certain HIV-1 strains (31).

HIV-1 can bind to resting $CD4^+$ cells, but subsequent T-cell activation is required for viral entry (36). Growth or differentiation factors may also enhance HIV-1 replication in monocytes (22, 37). However, the preferential growth of isolates from brain and lung compared to those from blood reported here occurred in the presence or absence of growth factors.

The HIV-1 envelope has been implicated as playing a critical role in monocyte tropism (37). In the current study, we were unable to demonstrate specific sequences in *env* of clones derived from brain or lung isolates compared to blood isolates that might be responsible for monocyte tropism. However, very limited sequence alterations may significantly

alter cell tropism (38). Furthermore, distinctive sequences in *nef* or the 3′ long terminal repeat were not found in clones derived from brain or lung isolates compared to those derived from blood isolates (unpublished data).

These findings might suggest that the clones do not reflect the biologically important variants in the primary isolates (39). However, the similarities in structure of multiple different clones from each isolate and sequences derived directly from PCR products of fresh tissues would argue against this possibility. Alternatively, monocyte tropism may be based at least partially on determinants other than *env*.

Truncated Envelope Products. The discovery of termination codons in *env* of each of these clones is intriguing. Since these termination codons are within or N-terminal to the membrane-spanning domain (Fig. 3), it was predicted that the isolates from which these clones were derived would be noninfectious, and functional studies in T lymphocytes confirm this hypothesis (unpublished findings). However, the presence of these termination codons in all the clones suggests that they are representative of the predominant virus strains present *in vivo*. Moreover, their demonstration in patient DNA by PCR amplification discounts the possibility that they arose in tissue culture. A helper virus present as a minor strain could be present *in vivo* or during the initiation of virus isolation in tissue culture that would allow propagation of a strain encoding a truncated envelope product.

It is possible that the HIV-1 strains isolated here are similar to the immunodeficiency-inducing strains of feline and murine leukemia viruses (40, 41), which are also defective. Termination codons have also been described in the portion of *env* encoding TM in other strains of HIV-1, simian immunodeficiency virus, and human T-lymphotropic virus type I (42–44).

Limited Sequence Heterogeneity. This study is notable in that only limited heterogeneity is present among molecular clones of the *env* gene derived from this clustered HIV-1 outbreak. Previous studies have documented (10) amino acid sequence differences in envelope of 1.4–27% between cultured isolates of unrelated patients. The clones described here display 15–27% amino acid differences compared to published sequences of unrelated isolates (10). Conserved and hypervariable regions in the clones described here reflect the general pattern of diversity seen with other published sequences (Table 1, Fig. 3). The CD4 binding region of SU (30) demonstrates 7.3% diversity of amino acid sequences compared to clone HXB2, whereas previously sequenced clones (10) demonstrate 4.9–19.5% amino acid changes compared to HXB2.

The conservation of cysteine residues between clone I-BL2 and previously sequenced clones (10) provides further support for the preservation of tertiary conformation of envelope. Furthermore, 22 of 24 potential N-glycosylation sites are preserved in I-BL2.

The current study of a transfusion-related HIV-1 outbreak compares molecular clones derived from three tissue sources from three patients over 3.5 years. Among studies of related isolates, the current investigation demonstrates significantly less heterogeneity (0.02% nucleotide and 0.01% amino acid changes) than has been noted in previous publications (11, 12).

There are several possible explanations for the discrepancy between the current findings and those previously described (11, 12, 39). (*i*) It is possible that the viral strains or clones reported here are not independent isolates. However, this is highly unlikely since the isolates were cultured at different times and by different investigators. The last viral strain and two clones obtained from it were isolated after completing all other work in this study. The viral strains also demonstrate different biological activities (Fig. 2). A clone with a distinct sequence was obtained at the same time from an unrelated isolate. Furthermore, PCR data from uncultured tissue samples of these patients confirm the close similarity of HIV-1 sequences.

(*ii*) It is possible that prolonged passage in PBMCs or cell lines *in vitro* may select for HIV-1 isolates with greater sequence diversity (39). Previous studies have analyzed at least some clones obtained by passage over several months in cell lines (11). The current study abbreviated cultures and used only PBMCs to reduce selection biases.

(*iii*) The limited sequence heterogeneity in the isolates described may be related to clinical features of these patients. Isolates from individuals neonatally infected may demonstrate less sequence variation due to failure of these individuals to mount an immune response and exert selective pressures on the virus. However, the current study includes isolates from the infected adult blood donor when he was asymptomatic and when he had ARC. It is possible that a greater level of sequence heterogeneity may occur with the development of progressive symptoms. Alternatively, the greater level of sequence variation found from clones obtained from the infected laboratory worker may be related to the possibility that he was infected with multiple different viruses simultaneously (12).

(*iv*) Truncation of the envelope products in these clones may shield these sequences from a putative selection pressure for sequence heterogeneity exerted by the host immune response, especially if a functional envelope product is produced by a helper virus.

(*v*) It is possible that the rate of envelope sequence change is itself variable, achieving levels of <0.1% in some patients and 3.0% in other patients. It is possible that this cluster contains a predominant infectious immunodeficiency-producing isolate replicating in a permissive environment.

The current data suggest that previous studies may have overestimated the level of sequence heterogeneity attained *in vivo* or that such heterogeneity is not an essential feature of HIV-1 infection. Thus, sequence variation may not be required for disease progression and may at least in part indirectly reflect host factors or *in vitro* phenomena. This study also suggests that the virus may not be mutating to a more infectious form but that host factors may be responsible for accelerating virus replication and disease progression.

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