Compartmentalization of Surface Envelope Glycoprotein of Human Immunodeficiency Virus Type 1 during Acute and Chronic Infection

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Human immunodeficiency virus type 1 is characterized by extensive genetic heterogeneity. Having previously demonstrated that, in the peripheral blood, the initial viral population is more homogeneous than at subsequent stages of infection, we have extended our studies to tissue samples, allowing comparisons between viral populations in peripheral blood and tissues during both the acute and chronic stages of infection. We found that homogeneity in gp120 sequences during the acute infection phase is not just restricted to the peripheral blood but also extends to other tissue compartments. However, in chronically infected individuals, heterogeneous and distinct viral populations were found in different compartments. We therefore conclude that the dominant and homogeneous viral population observed during the acute infection phase is likely to infiltrate lymphoid tissues and form the genetic bases for subsequent diversification. It is therefore likely that the compartmentalization of viral sequences observed in chronically infected patients reflects a gradual diversification of a common dominant viral variant rather than the preferential migration of distinct viral populations to different tissue compartments at the beginning of infection.

A striking feature of human immunodeficiency virus type 1 (HIV-1) infection in vivo is the rapid generation and turnover of viral variants, resulting in a high degree of sequence diversity both within and between infected individuals (1, 6, 8, 11, 18, 23, 26, 27, 29, 39, 47–49, 53). While the time course of the disease seems slow, the generation of different viral variants in vivo is rapid and persistent throughout the course of infection. It is generally believed that HIV-1 infection begins with a relatively homogeneous population (7, 25, 29, 33, 45, 56, 57). However, as the disease progresses, divergent viral variants with distinct genetic sequences emerge, probably as a result of selective pressures imposed by the immune system, therapeutic regimens, or preferential tropism for certain target cells (3–5, 17, 26, 36, 38, 42, 56). Thus, an increasingly complex and heterogeneous viral population is almost exclusively found in chronically rather than acutely infected patients. Over time, these viral variants often result in the formation of distinct quasispecies and are capable of localizing themselves in various tissue compartments (9, 10, 15, 17, 21, 22, 31, 32, 43–46, 58). Understanding more about the unique nature of viral diversification, dissemination, and compartmentalization during the acute and chronic phases of infection is critical to our understanding of viral pathogenesis and the development of an effective drug regimen against those variants residing in various tissue compartments.

Numerous reports have provided evidence of distinct HIV-1 variants in vivo in chronically infected patients, as indicated by their envelope sequences. Viral variants recovered from different organ- or tissue-specific viral populations that arise during the course of HIV-1 infection. The second theory is based on the parallel evolution of viral variants. Upon infection, a homogeneous viral population infiltrates all tissues. However, over the course of infection, tissue-specific selection pressures favor minor independent variants, which will actively replicate in accordance with their unique environment and thereby produce a subpopulation distinct from those of neighboring compartments.

Our present study sought to evaluate these two hypotheses by studying variation in the gp120 sequences in tissues and peripheral blood from both acutely and chronically infected individuals. We believe that this is the first study of this kind to

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evaluate the genetic diversity and relatedness of HIV-1 in both peripheral blood and tissues during the acute infection phase. Our results show that homogeneity in gp120 sequences during the acute infection phase is not just restricted to the peripheral blood but also extends to various tissue compartments. In contrast, in chronically infected individuals, distinct viral populations were found in different compartments. We therefore conclude that the dominant homogeneous viral population observed during the acute infection phase is likely to infiltrate lymphoid tissues and thereby form the genetic basis for further diversification during the subsequent stages of infection. The unique selection pressures that individual compartments are believed to experience will encourage the best-suited local variant to replicate with a higher frequency over the course of the infection. The compartmentalization of viral sequences observed in chronically infected patients therefore reflects a gradual diversification from a common dominant viral variant rather than the preferential migration of distinct viral populations to different tissue compartments at the beginning of the infection.

MATERIALS AND METHODS

 Patients. Eleven patients ranging in age from 30 to 54 years were recruited for the study (Table 1). Five of them were enrolled in treatment protocols during the acute infection phase and selected for this study because of their complete suppression of HIV-1 in plasma under the prior treatment and their full complement of CD4+ T lymphocytes. Most of these acutely infected patients demonstrated clinical symptoms consistent with acute infection. All acutely infected individuals were enrolled within 90 days of documented seroconversion. The remaining six patients were enrolled in treatment protocols at least 90 days after infection and were therefore determined as chronically infected. At baseline, these patients had a mean CD4+ T lymphocyte count of 308×10^3 cells/mm^3.

TABLE 1. Demographic, clinical, and virologic profiles of study subjects

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)/gender/risk factor</th>
<th>Baseline plasma HIV-1 RNA (no. of copies/ml)</th>
<th>Baseline CD4 count (no. of cells/mm^3)</th>
<th>Treatment regimen*</th>
<th>Infection phase at initiation of therapy*</th>
<th>Tissue sample collected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33/M/homosexuality</td>
<td>611,566</td>
<td>432</td>
<td>AZT/3TV/Ind</td>
<td>Acute</td>
<td>Tonsil</td>
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<td>2</td>
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<td>387</td>
<td>AZT/3TV/Ind</td>
<td>Acute</td>
<td>Tonsil</td>
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<tr>
<td>3</td>
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<td>49,737</td>
<td>544</td>
<td>AZT/3TV/Ind</td>
<td>Acute</td>
<td>Tonsil</td>
</tr>
<tr>
<td>4</td>
<td>38/M/homosexuality</td>
<td>38,940</td>
<td>290</td>
<td>AZT/3TC/Rit/Saq</td>
<td>Acute</td>
<td>Rectum</td>
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<td>5</td>
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<td>284</td>
<td>AZT/3TC/ABC/Amp</td>
<td>Acute</td>
<td>Rectum</td>
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<td>314</td>
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<td>Chronic</td>
<td>Rectum</td>
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<td>Chronic</td>
<td>Rectum</td>
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<td>302</td>
<td>AZT/3TC/ABC/Amp</td>
<td>Chronic</td>
<td>Rectum</td>
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<td>75</td>
<td>AZT/3TC/Nel</td>
<td>Chronic</td>
<td>Rectum</td>
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<tr>
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<td>30/M/homosexuality</td>
<td>126,425</td>
<td>12</td>
<td>AZT/3TC/ABC/Amp</td>
<td>Chronic</td>
<td>Rectum</td>
</tr>
</tbody>
</table>

* M, male.

† Determined by the Amplicor assay.

‡ Rit, ritonavir; Saq, saquinavir; Nel, nelfinavir.

§ Acute, treatment initiation within 90 days of documented seroconversion.

∥ All rectal samples were collected before initiation of therapy, whereas tonsil samples were collected 12 to 24 months after initiation of therapy.

The Committee for the Protection of Human Subjects from Research Risks at The Rockefeller University prior to any endoscopic or biopsy procedures. Whole-blood samples and flexible sigmoidoscopic biopsies from noninflamed rectal tissue were collected simultaneously before the initiation of highly active antiretroviral therapy. The endoscopic mucosal specimens collected in the study contained primarily the surface epithelium and lamina propria. Tonsillar biopsies were collected from acutely infected patients 12 to 24 months after initiation of therapy. PBMC were isolated from the whole blood by Ficoll-Hypaque separation (52, 55). Tissue samples were minced with scissors and then treated with 0.5 mg of collagenase (Sigma Chemical Company, St. Louis, Mo.) per ml at 37°C until the tissue was completely digested. Cell suspensions were obtained by filtration of collagenase-treated tissue samples through a 100-μm-pore-size nylon cell strainer (Becton Dickinson, San Jose, Calif.). Isolated PBMC and tissue cells were then used for viral isolation and genomic DNA extraction (see below). Viral RNA was extracted from the plasma; this was followed by cDNA synthesis and PCR amplification in accordance with previously published protocols (52, 55).

Virus isolation from clinical samples. Approximately 5×10^5 cells of patients' PBMC or cells isolated from rectal biopsies were cocultured with 5×10^5 phytohemagglutinin-stimulated normal donor PBMC as previously described (51, 54). The culture supernatant was monitored for p24 production on days 0, 4, 7, 10, and 14 with a commercial enzyme immunoassay (Abbott Laboratories, Abbott Park, Ill.). A culture was considered positive if the p24 value was above a cutoff of 40 pg/ml. Virus titers were determined on PBMC to determine the 50% tissue culture-infective dose (TCID_{50}).

Determination of viral phenotype and coreceptor usage. The phenotype of viruses was determined by testing their ability to generate syncytia in MT-2 cells as described previously (51, 54). In brief, MT-2 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, L-glutamine, and antibiotics and split twice a week. For infection assays, 2×10^5 cells were incubated with 100 TCID_{50} of each viral isolate for 4 h. Unbound viruses were removed by two washes in culture medium. The culture supernatant was tested for HIV-1 p24 antigen on days 0, 4, and 7 postinfection. MT-2 cell cultures were evaluated by light microscopy for syncytium formation.

U87MG.CD4 cell lines stably transfected with the CCR1, CCR2b, CCR3, CCR5, or CXCR4-encoding gene and HOS.CD4 cells stably expressing BOB/gpr15 and Bonzo/STRL33 were kindly provided by D. Littman (Skirball Institute for Molecular Medicine, New York, N.Y.) (51, 54). These cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (1 μg of puromycin [Sigma Chemical Company] per ml), 100 μg of neomycin [G418; Sigma Chemical Company] per ml, and 250 μg of hygromycin [Sigma Chemical Company] per ml) and split twice a week. For infection experiments, 10^5 cells were incubated with 100 TCID_{50} of each isolate for 4 h and unbound virus was removed by three washes in culture medium. The cultures were examined microscopically for the formation of multinucleated foci, and the supernatant was analyzed for the presence of p24 antigen.

PCR amplification, length polymorphism, sequencing, and sequence analysis of HIV-1 gp120. The entire procedure for PCR amplification, length polymorphism, sequencing, and sequence analysis of HIV-1 gp120 has been previous
Comparison of sequences in the surface envelope glycoprotein of HIV-1 between peripheral blood and tissues during the acute and chronic phases of infection. To analyze viral populations in both peripheral blood and tissues in greater detail, extensive sequencing was conducted in all five acutely infected and six chronically infected individuals. A total of 380 sequences from the C2-V3 region were obtained from both PBMC and tissues, 230 of which were from PBMC and the remaining 150 of which were from tissues. All of these sequences were obtained as single molecules through limiting dilution prior to PCR to avoid errors generated during the amplification step (40). The most notable result of the sequencing is the confirmation of the results obtained by length polymorphism analysis. Figure 2 summarizes the consensus sequences obtained from PBMC, tonsillar, and rectal samples. In five acutely infected individuals (patients 1 to 5), the sequences from PBMC and tonsil or rectum tissue are identical, consistent with the results obtained from the length polymorphism studies (see above). In contrast, significant differences were found between PBMC and rectal samples in all chronically infected patients (patients 6 to 11). In patients 6, 7, and 8, large numbers of amino acid changes were found outside the V3 loop; in patients 9, 10, and 11, changes were also found inside the V3 loop sequence. The impact of these changes on the viral replication kinetics, phenotype, and coreceptor usages is relatively minimal (see below). An analysis of the genetic diversity within and between different compartments has also been conducted. It has been found that the average genetic diversity in the peripheral blood (0.38% ± 0.38%) is significantly smaller (P < 0.5) that that in tissues (0.58% ± 0.58%). In addition, the average genetic distance between sequences from the peripheral blood and tissue is approximately 6.3% ± 3.2%, which is roughly 10- to 20-fold greater than that within the same compartment. The average interpatient diversity, however, is about 15 to 20%.

Further analyses of sequences obtained from the chronically infected individuals were conducted by using several phylogenetic approaches. Figure 3 depicts the neighbor-joining trees generated for all six patients with sequences from both PBMC and rectal tissue. Individual sequences are represented by patient codes. For clarity of tree presentation, only selected sequences are shown for each patient. The horizontal branches reflect the relative genetic distance between different sequences. The most obvious and consistent result of these analyses is that sequences from each individual clustered together without contamination between patients or with HIV-1 laboratory strains such as HXB2, OYI, JRFL, and RF (Fig. 3). In addition, sequences from PBMC and rectal tissue are more similar within each compartment, as reflected by their tight clustering on the phylogenetic tree (Fig. 3). In contrast, sequences obtained from different compartments are significantly different, as demonstrated by the distinct clustering of sequences separated by a long branch length on the tree (Fig. 3). This observation supports the findings of length polymorphism analysis that there are significant differences between peripheral blood and rectal tissue in all six chronically infected patients.

Isolation and phenotypic characterization of viruses from the PBMC and rectums of chronically infected individuals. To study whether the genetic differences observed between virus
samples obtained from peripheral blood and rectal tissue have differing viral phenotypes, we isolated viruses from all six chronically infected patients by using the same PBMC and rectal cells as were used for the genetic analysis. We then characterized viral phenotypic properties by several means. Firstly, we tested the ability of viral isolates to replicate in PBMC. Approximately 100 TCID50 of each isolate was used to inoculate 2 × 10^6 phytohemagglutinin-stimulated PBMC, and the production of p24 antigen in the culture supernatant was measured on days 0, 4, 7, 10, and 14. All viral isolates, except one from the PBMC of patient 10, replicated in PBMC with similar kinetics, without significant differences between those from peripheral blood and those from rectal cells (Fig. 4).

To assess the ability of virus to generate syncytia in MT-2 cells and to evaluate their coreceptor usage, isolates were inoculated into MT-2 cells and U87MG.CD4 cells stably transfected with the chemokine receptor CCR1, CCR2b, CCR3, CCR5, or CXCR4. All viral isolates failed to generate syncytia in an MT-2 cell culture and failed to use coreceptors other than CCR5 (data not shown). All of these isolates therefore fit into the category of R5 or non-syncytium-inducing viruses.

**DISCUSSION**

In this work, we have carried out an extensive investigation of the genetic polymorphism in the gp120 sequences of HIV-1 in peripheral blood and tissues during the acute and chronic infection phases. We believe that this is the first study of this kind to evaluate the genetic diversity and relatedness of HIV-1 in both peripheral blood and tissue during the acute infection phase. Viral sequences from both peripheral blood and tissues were analyzed by using multiple techniques. The profiles of length polymorphism in the V1-V2 and V4-V5 regions of gp120 gave a single and uniform peak in both the peripheral blood and tissues of all acutely infected individuals. More importantly, these profiles are identical in all tissues during the acute infection phase. This inferred homogeneity was confirmed by sequence analysis. This finding suggests that homogeneity of viral sequences during the acute infection phase is not just restricted to the peripheral blood but extends to most tissue compartments. Explosive viral replication during the acute infection phase therefore populates all body compartments with a dominant viral variant, discounting the likelihood that different variants preferentially target different tissue compartments during the acute infection phase. This observation is consistent with previous findings based on peripheral blood sampling (7, 25, 29, 56, 57) that during the acute infection phase, the viral population is relatively homogeneous compared with that in the later stages of infection.

In contrast to what was observed in acutely infected patients, multiple peaks with different lengths of both the V1-V2 and V4-V5 regions of gp120 gave a single and uniform peak in both the peripheral blood and tissues of all acutely infected individuals. More importantly, these profiles are identical in all tissues during the acute infection phase. This inferred homogeneity was confirmed by sequence analysis. This finding suggests that homogeneity of viral sequences during the acute infection phase is not just restricted to the peripheral blood but extends to most tissue compartments. Explosive viral replication during the acute infection phase therefore populates all body compartments with a dominant viral variant, discounting the likelihood that different variants preferentially target different tissue compartments during the acute infection phase. This observation is consistent with previous findings based on peripheral blood sampling (7, 25, 29, 56, 57) that during the acute infection phase, the viral population is relatively homogeneous compared with that in the later stages of infection.

**FIG. 1.** Length polymorphism of the proviruses in PBMC in comparison with those isolated from tonsillar or rectal samples during the acute (a) or chronic (b) phase of HIV-1 infection. V1-V2 region, red; V4-V5 region, blue. The size of each peak is indicated by the scale at the top of each panel.
FIG. 3. Unrooted neighbor-joining tree (Kimura two-parameter model) depicting the evolutionary relationship between viruses from PBMC and tissues of six chronically infected patients. Individual sequences are represented by patient codes at the ends of branches, which are drawn to scale so that the relatedness between different sequences can be readily assessed. P; PBMC; R; rectum. For each individual, only a selected number of sequences are shown in the tree for clarity of presentation. Laboratory HIV-1 strains such as HXB2, OYI, JRFL, and RF were included as controls.
V4-V5 regions were identified in peripheral blood and tissues in chronically infected patients. Significant differences between the peripheral blood and tissues in the profile of length polymorphism and sequences were also found in these patients, indicating that distinct viral populations reside in the different compartments. In the context of what we have observed in acutely infected individuals, this finding suggests that the compartmentalization of viral sequences observed here and elsewhere (2, 9, 10, 12, 14, 15, 17, 20–22, 24, 30–32, 34, 35, 37, 43–46, 58) must have occurred at a later point in infection. Furthermore, phenotypic characterization of viruses isolated simultaneously from the peripheral blood and rectal samples of the six chronically infected patients showed them to be similar in replication kinetics and coreceptor usage despite the significant genetic differences between their gp120 sequences. It is probable that the observed amino acid differences do not have a significant impact on these viral phenotypic characters or that current assays are not sensitive enough to discern those differences. In any case, homogeneity in viral sequences during the acute infection phase in both peripheral blood and tissues strongly suggests that a dominant variant has infiltrated all of the tissue compartments. Once this homogeneous viral population populates a particular tissue compartment, it subsequently diversifies and becomes distinct from the virus in other compartments, most likely as a result of the different selective environments in different tissue compartments. The different viral populations in the peripheral blood and tissues of chronically infected patients are therefore more likely to reflect gradual diversification from a common dominant viral variant rather than preferential targeting of distinct viral populations at the beginning of the infection.

One may only speculate as to the mechanism underlying the homogeneous viral population that exists during the acute infection phase. The simple laws of population genetics dictate that when a highly complex mixture of species expands to fill a new environment by uncontrolled replication, genetic polymorphism is lost, sometimes completely (13, 16, 41, 50). These founder effects are a natural consequence of uncontrolled expansion, are stochastic in nature, and (perhaps surprisingly) do not require any selective force to deliver a monotypic population (13, 16, 41, 50). It is also possible that homogeneity is a result of selective constraints imposed on the incoming viral population either by replication competition among different variants or by the external environment in the form of immune surveillance and the availability of target cells. As a result, the fastest-growing and/or best-suited viral variant
comes to dominate the entire human body during the acute infection phase. Part of these selection pressures could act on the envelope gene, which could help explain the observation that the majority of the viruses isolated during the acute infection phase use CCR5 as a coreceptor. However, selective pressure on the envelope-encoding gene alone cannot explain the whole story, in particular in those acutely infected women in whom relatively divergent rather than homogeneous viral populations have been identified during the acute infection phase (28). Differences in the hormonal regulation or the availability, type, and number of target cells between the male rectal and female vaginal tracts may offer some explanations for the observed differences. It is probable that both viral and host factors play critical roles in the emergence of a homogeneous population during the acute infection phase.

In summary, our study demonstrates that homogeneity in gp120 sequences during the acute infection phase is not restricted to the peripheral blood but extends to the tonsils and rectum and possibly to all tissue compartments. The dominant strain during the acute infection phase is therefore likely to form the genetic basis for the subsequent diversification of HIV-1 in various tissue compartments during the later stages of infection. Our previous findings that viral populations continue to diverge from the initially dominant population during the course of infection (18, 53) strongly support this notion. We conclude that the compartmentalization of viral sequences observed in chronically infected patients reflects a gradual diversification from a common dominant viral variant at seroconversion rather than a variety of distinct viral populations migrating to different tissue compartments at the start of the infection. Finally, our findings here and elsewhere argue strongly for early treatment intervention before these viruses become divergent and hence more likely to develop both drug resistance mutations and other mutations associated with immune evasion.

ACKNOWLEDGMENTS

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