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Nonhomogeneous Distribution of Human Immunodeficiency Virus Type 1 Proviruses in the Spleen

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A nonhomogeneous spatial distribution of human immunodeficiency virus type 1 proviruses in an infected spleen was observed. Antigenic stimulation of infected cells might explain this partition.

The high degree of sequence variability that exists between different isolates of human immunodeficiency virus type 1 (HIV-1) (7) or within a single infected individual (2, 6, 9) poses several major problems, not least of which is the development of effective methods of immunization against the virus. It is not known whether this genetic heterogeneity is a cause or a consequence of immunodeficiency or merely parallels the disease process. Because of the quasispecies nature of RNA viruses, it is difficult, especially for HIV, to even define a single sequence characteristic of an isolate. It is therefore possible that variants adapted for certain tissues (3, 8) or microenvironments may be selected from the myriad of genomes present in an individual during the course of infection. A comparison of brain and spleen tissue from three infected children showed that both brain- and spleen-derived HIV-1 V3 sequences formed distinct quasispecies (3). A different study described sequence differences from peripheral blood- and brain-derived sequences (8). The brain may constitute a reservoir for HIV because of poor immune surveillance. In contrast, the spleen is a secondary lymphoid organ and a center of immune responses to blood-borne antigens. Thus, it is possible that local antigenic stimulation of antigen-specific lymphocytes bearing HIV proviruses introduces a spatial inhomogeneity in the spleen.

As opposed to our previous work, which focused on the evolution of HIV quasispecies over time (2, 6, 9) or comparisons of the same region from several patients (10), the present study analyzed HIV quasispecies derived from different fragments of the spleen, as well as the peripheral blood, of an infected individual.

This patient was an asymptomatic carrier (Centers for Disease Control class II) with a thrombopenia that was sensitive to corticoids yet insensitive to androgens or zidovudine. Splenectomy was performed when the patient's $CD4^+$ T-cell count was 150/μl. There was no splenomegaly. A blood sample was taken on the same day. Five spleen fragments of approximately 2 to 4 cm^3 each were taken. They represented 80% of the organ. Cells were recovered by abrading the fragments with a scalpel. Spleen mononuclear cells and peripheral blood mononuclear cells were purified on Ficoll-Hypaque gradients. Total DNA was extracted from aliquots of 500,000 cells.

Two aliquots (P1 and P2) of PBMCs were analyzed, as were two aliquots derived from the same spleen fragment (R21 and R22). The other samples came from spleen fragments referred to as R12, R41, R51, and R61. To maximize discrimination between data sets, proviral sequences corresponding to the V1 and V2 hypervariable regions within the gp120 sequence were analyzed.

The proportion of HIV-1-infected mononuclear cells was analyzed by limiting dilution and nested polymerase chain reaction (PCR). Serial dilutions of spleen mononuclear cells were made with murine X63 myeloma cells as the carrier. The final number of cells per dilution was 10,000. Cells were directly lysed in PCR tubes with TPK buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% Tween 20, 100 μg of proteinase K per ml). After 1 h at 56°C, the proteinase K was inactivated by boiling for 10 min. The PCR was performed under standard conditions (2.5 mM $MgCl_2$, 100 pmol of primer, 100 μM each deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase [NBL]). The outer primer pair consisted of LV15 (5'GCCA CACATGCCTGTGTACCCACA3') and LV13 (5'CTTTA GAATGCGAAAACCAGCCG3'), while the inner primer pair consisted of SK122 and SK123 (4). All primers mapped within the highly conserved regions flanking V1 and V2. Thermal cycling parameters for the first round of 35 cycles were as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min, and then a 10-min extension time at 72°C. Ten percent of the first round product was amplified with the inner primer pair for another 35 cycles under the same conditions. Six dilutions, each replicated 10 times, were studied for each sample. The PCR products were electrophoresed and Southern blotted. The

TABLE 1. Sequence distribution in different samples

Sample	PCN[a]	% of indicated sequence in sample[b]															
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	Q	R
P1	ND	40	20	10	5	5	5											
P2	ND	35	15					10	5							5		
R12	30	25	5								15	20	10	10			5	
R21	100	40	5															
R22	100	25	5					5	5	10	5							
R41	180	25	30		5	5	5				5				10			
R51	40	25	25					20									5	10
R61	ND	15	50					10								5		

[a] PCN, proviral copy number per 150,000 spleen mononuclear cells; ND, not determined.

[b] Only sequences common to two or more clones are shown. The relationship between the sequence designation and the V1-V2 *env* sequence is described in the legend to Fig. 1.

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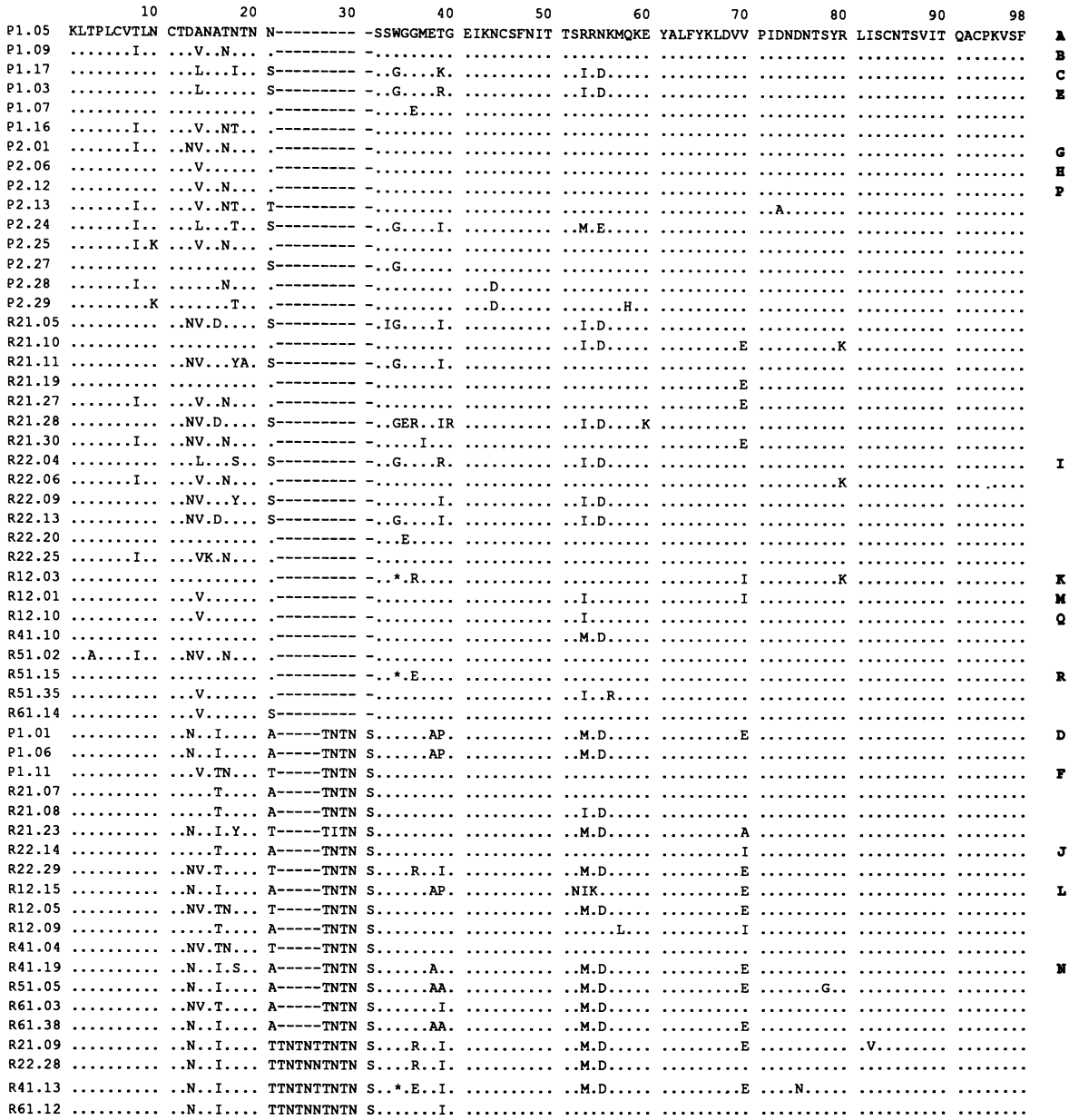

FIG. 1. Alignment of all of the distinct V1-V2 amino acid sequences. All sequences, shown in the one-letter code, are aligned with P1.05, the most frequent among the 160 sequences. Only differences are shown. Sequence identity is indicated by a dot. The length polymorphism in the V1 region is indicated by gaps (–) in the shorter sequences. An asterisk indicates an in-phase stop codon. Sequences with the prefix P were derived from the peripheral blood mononuclear cell sample, while those with the prefix R were derived from spleen fragments. R21 and R22 represent sequences from different aliquots of the same fragment. Letters on the right indicate sequences either common to more than one sample or present at a frequency of ≥10% in a single sample.

filter was probed with the ^{32}P-labelled SK129 oligonucleotide (4). The results were analyzed by using the Poisson distribution (5). The proportion of HIV-infected mononuclear cells varied approximately sixfold between spleen samples (Table 1).

The PCR products from a single 45-cycle amplification of 1 μg of sample DNA with the inner primers SK122 and SK123 were cloned (2) into M13 mp18 DNA. The recombinants were sequenced as described elsewhere (2). For all samples except R12 (see below), the amino acid quasispecies faithfully reflected the nucleic acid quasispecies, i.e., the major protein sequences were derived from identical nucleic acid sequences. The results are summarized in Table 1 and Fig. 1. The sequences common to two or more clones were designated A to R. Their proportions in the samples are shown in Table 1, and their sequences are given in Fig. 1. It was important to note the reproducibility of the results with both the samples from blood (P1 and P2) and those from the spleen (R21 and R22). Independent amplifications of cellular DNA from cells of the same sample yielded comparable quasispecies. Even though further sequencing identified more variants, the proportion of the major sequences re-

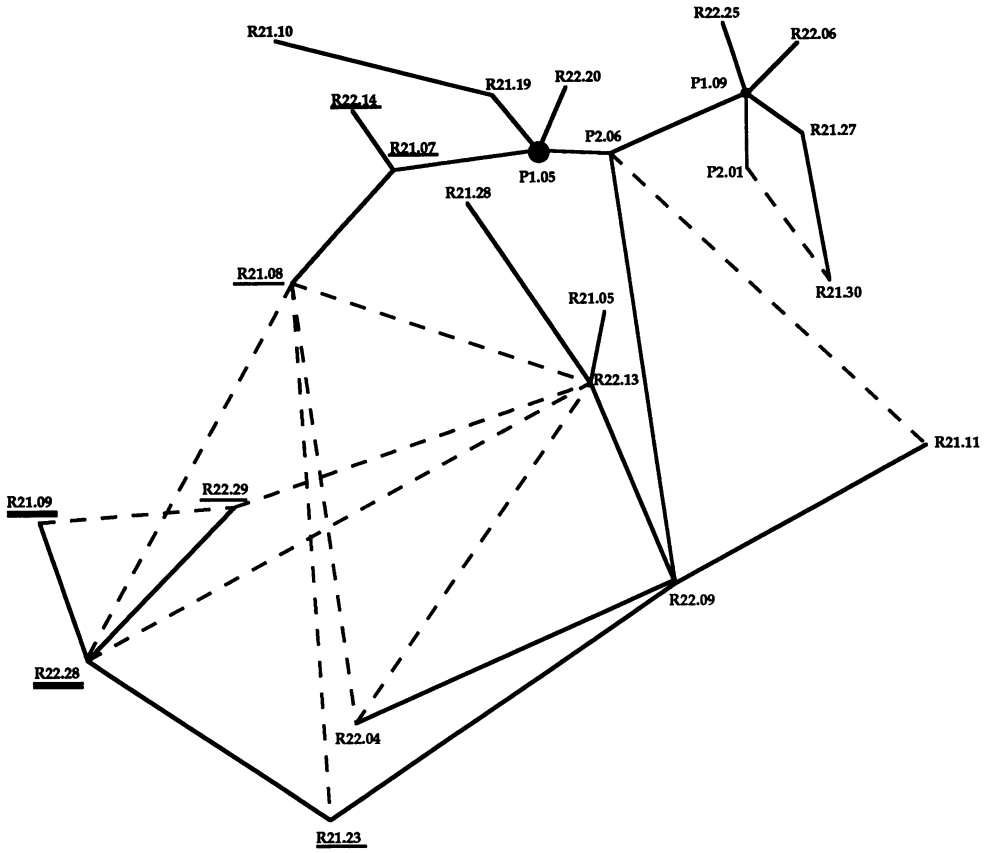

FIG. 2. Phylogenetic tree of spleen amino acid sequences from 40 clones (R21/22 series) derived from the same fragment. The line length reflects the number of substitutions between sequence pairs. Solid lines indicate the first tree found. Dotted lines indicate extra connections found by permuting the sequence that initiated the algorithm. Sequences A (P1.05) and B (P1.09), which are the only sequences common to R21 and R22, were located at two nodes and are indicated by black dots. Sequences bearing a single or double duplication are underlined or doubly underlined, respectively.

mained the same (±10%). The overall structure of the quasispecies did not vary, and 20 clones gave a good representation of the viral population in a sample.

Two sequences (A and B) were present in all the samples studied. However, their proportions varied from 15 to 40% and from 5 to 50%, respectively, depending on the fragment. While the structure of the spleen quasispecies R41 and R51 reflected those from the blood (P1 and P2), others (R12, R21/22 [a single quasispecies from samples R21 and R22], and notably R61) were different. Thus, there was a spatial distribution of HIV-1 proviruses in the spleen which could be detected despite the infected PBMCs pervading the organ.

Figure 1 presents all the distinct amino acid sequences composing the eight studied samples. All sequences were aligned with the P1.05 (A) amino acid sequence, which represented the major form (30%) among the 160 sequences. There was a degree of sequence homology which suggested that they derived from a single infection (10). V1 was more variable than V2 and had a length polymorphism. This was exemplified by sequence R21.09 (TNTNT TNTNT TNTNS), in which there appeared to have been at least two duplications (underlined). Similar examples could be found in all the samples studied.

Three sequences (R12.03, R41.13, and R51.15) were defective, having an in-frame stop codon at position 34. R12.03 (sequence K) represented 20% of the R12 sample (Table 1). The presence of substantial proportions of defective HIV genomes has been noted elsewhere (6, 9). However, at the nucleic acid level, all four clones corresponding to R12.03 were identical, while those making up the A form all differed from each other by silent base substitutions; i.e., at the nucleic acid level, a defective genome was dominant. It is possible that the R12.03 population of genomes was expanded because of antigenic stimulation of a cell harboring this provirus. Circulating lymphocytes pass through the spleen in as little as 6 h. Antigen-specific T lymphocytes are sequestered in the T-cell-dependent areas of the splenic white pulp. There they may become activated by contact with antigen. Extensive lymphocyte proliferation follows, with reentry of short-lived effector cells into circulation within 3 days. As the blood and spleen samples were obtained on the same day, it is possible that, given the lag between sequestration of antigen-specific T cells and reentry of the effector cells into circulation, the blood sample does not reflect all the events that took place in the spleen.

If a defective genome could be expanded by cellular replication rather than reverse transcription, it would obviate the need for helper virus. In addition, the discrepancies between proviral distributions in the different spleen fragments may reflect a spatial distribution of antigen and consequently T-cell activation within spleen fragments, par-

ticularly for samples R12, R21/22, and R61. In a different system, clonal expansion has been observed in a study of the Vβ repertoire of murine T-cell receptor genes in response to a protein antigen (1).

The extent of sequence variation prompted a phylogenetic analysis of the sequences. We looked for minimal spanning trees, in which leaves as well as internal nodes correspond to observed sequences (4). Prim's algorithm (11) was used for that purpose. This procedure can lead to different tree topologies of the same value depending on the sequence used to initiate the algorithm. In order to evaluate the stability of the tree topology, the procedure was initiated with each sequence in the set. A number of alternative topologies which varied considerably with respect to the sequence set were observed. In the case of P1 and P2, only one extra edge was found. The tree for R21/R22-derived sequences (i.e., from the same spleen fragment) is shown in Fig. 2. The first tree found is shown, as are 10 extra edges corresponding to alternative topologies. Despite these reservations, a number of sequences, particularly forms A (P1.05) and B (P1.09), could be found at nodes, suggesting sequential evolution with the accumulation of variants. In general, the sequences with duplications clustered together. The double duplication probably arose from an existing direct repeat. Their apparent stability in HIV contrasts with the predilection for deletions during single-cycle replication of spleen necrosis virus, an oncoretrovirus (12). However, only when comparable data are available for HIV will it be possible to reliably compare these different retroviruses.

The possibility that proviral populations can be altered by antigenic stimulation of lymphocytes would help to explain the fluctuations of HIV quasispecies. It suggests that the expansion of a given sequence may have nothing to do with the fitness of a given genome, with the case of the defective R12.03 genome being an extreme example. Rather, the outgrowth of a variant might be due to the chance encounter, mediated by the T-cell receptor, of an antigen and a latently infected T lymphocyte.

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