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Divergent Patterns of Progression to AIDS after Infection from the Same Source: Human Immunodeficiency Virus Type 1 Evolution and Antiviral Responses

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The rate of progression to AIDS in human immunodeficiency virus type 1 (HIV-1)-infected individuals is determined by a complex series of interactions between the host and virus. Here we evaluate virologic properties and host responses in two men near-simultaneously infected with HIV-1 from the same sexual partner—one individual progressed to AIDS in less than 2 years, and the other remains asymptomatic 3 years postinfection. Distinct neutralizing antibody and cellular immune responses were evident, with the slower progressor exhibiting generally stronger and broader responses, except for cytotoxic T-lymphocyte responses early in infection. Virtually identical, homogeneous virus populations were found in both patients in the first sample obtained; however, a second unrelated HIV-1 virus population was also found in the fast progressor. Whether the second population emanated from an additional source of infection or the two were transmitted from the original source could not be determined. The virus population in the slower progressor turned over and diversified rapidly, whereas both virus populations in the rapid progressor evolved at a much slower rate. In addition, the character of mutational changes underlying these diversities appeared to be distinct, with a bias for diversifying selection developing in the slower progressor and a reciprocal bias towards purifying selection maintained in both populations in the fast progressor. Thus, the rapid evolution that is a hallmark of HIV replication may be a reflection of strong host resistance against emerging virus variants and a longer period of asymptomatic infection. Furthermore, rapid progression was not linked to a collapse of any appreciable immune response following attainment of some threshold of antigenic diversity but rather to a failure to drive this diversification and a condition of relatively unimpeded expansion of variants with optimized replicative capacity within the host.

Upon primary infection with human immunodeficiency virus type 1 (HIV-1), a large burst of virus replication occurs, followed by the onset of detectable host cellular and humoral immune responses (4, 7, 10, 26, 42, 55, 79, 81–83). These responses, particularly cytotoxic T lymphocytes (CTL), are thought to play an important role in the diminution of the initial burst of viremia (3, 42), although this theory is controversial (64). The rate of disease progression in a given individual is then likely determined by a complex interaction between the host and an increasingly diverse viral population (7, 26). The mechanisms leading to the continual loss of $CD4^+$ T lymphocytes, the eventual loss of overall T-cell homeostasis (48), and ultimate progression to clinical AIDS are poorly understood, although several hypotheses or models have been put forward (53), including those focused on virulent variants (4, 8, 20, 25, 39, 56, 57, 62, 71, 76, 77), viral antigenic diversity (56, 57, 60, 61), host immunocompetence (85), and virus load and target cell availability (7, 27, 28), as well as the null hypothesis that inherent properties of the transmitted virus in a given host context determine the kinetics of disease progression (70).

Studies from animal model systems have indicated that AIDS-like syndromes result from and along with the appearance and fulminant replication of newly evolving or previously occult or transmitted virulent virus variants (18, 20, 58, 62). In humans, quantitative virus burden is strongly correlated with disease progression (28, 34, 51, 65). Qualitative aspects of the virus population, including in vitro cytopathogenic HIV-1 strains, have also been implicated in disease progression (9, 76, 77), as has loss of CTL responsiveness to viral antigens (38). However, the transition of virus phenotype from non-syncytium inducing to syncytium inducing has been associated with disease progression in some, but not all, cases of AIDS (13, 19, 24, 39), and in general the requirement for the evolution or transmission of virulent variants for AIDS pathogenesis in humans remains unclear (63). Nowak et al. (60) suggested that the accumulation of virus variants can eventually exhaust the capacity of the immune system to respond to additional variants; however, several studies have failed to support the general tenets of this hypothesis but have rather demonstrated that greater quasispecies complexity, and hence broadening antigenic diversity, is associated with a slower rate of $CD4^+$ cell decline and disease progression (16, 47, 80). Also linked to slow disease progression are high levels of CTL and low virus load (4, 53, 68), whereas defects within the accessory genes *nef* (11, 37) and *rev* (33) have been linked to a minority of cases of long-term asymptomatic HIV-1 infection. Heterozygosity for a functional macrophage-tropic HIV-1 coreceptor, CCR5, has also been reported to be associated with slower disease progression (12, 30).

To assess the relative contributions of host and virus to disease outcome, Wolinsky et al. (80), studying patients unmatched for virus source, as well as our group in this study on

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patients infected from the same source, have sought to integrate analysis of virologic properties and host responses to identify factors contributing to or determining the rate of disease progression. To control for virologic factors as much as possible, we examined the cases of two men, infected by a common source, who progressed to disease at different rates. We describe a multifaceted analysis of virus population dynamics and host immune responses in these individuals.

MATERIALS AND METHODS

Study subjects. Patients A and B are male homosexual partners who both experienced acute retrovirus syndrome 5 and 10 days, respectively, after a single sexual encounter with a third, HIV-infected man. During this encounter they reported multiple unprotected oral-genital and anal-genital contacts with the third person. Patients A and B were both negative for HIV by serologic assay performed 6 weeks prior to this sexual encounter, and they reported no other sex partners during the interval between negative testing and the encounter with the common-source HIV-infected person, as well as between onset of acute retroviral syndrome and initial sampling. Patient A, a 26-year-old Caucasian male, lost approximately 31 $CD4^+$ cells/mm^3 per year during the first 3 years of follow-up and remains largely asymptomatic, with minor skin complaints and intermittent depression. He has not had detectable p24 antigenemia (Fig. 1a). In contrast, patient B, a 38-year-old Caucasian male, progressed quickly, with a rapid loss of $CD4^+$ cells (175 per year of follow up) and has had persistent p24 antigenemia (Fig. 1a) and early onset of a series of clinical manifestations such as seborrhea, psoriasis, and *Pneumocystis carinii* pneumonia. He was diagnosed with AIDS (cytomegalovirus esophagitis) 1.5 years postinfection (PI) and has been treated with antiretroviral drugs since day 825 PI (Fig. 1a).

Quantitation of virus and proviral DNA. The number of infectious units per million peripheral blood mononuclear cells (PBMC) (IUPM) was determined by using the AIDS Clinical Trial Group (ACTG) consensus procedures (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Md., 1993). PBMC DNA was isolated by using the Isoquick Nucleic Acid Extraction kit (Microprobe, Garden Grove, Calif.) and proviral DNA was quantified by serial endpoint dilution (EPD). First-round PCR primers were ED5 (5′-ATGGGATCAAAGCCTAAAGCCATGTG, 6556-6581, HIV-1 HXB2) and ED12 (5′-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG, 7822-7792, HIV-1 HXB2); second-round primers were ES7 (5′-gtaaaacgacggccagtC TGTTAAATGGCAGTCTAGC, 7001-7020, HIV-1 HXB2; the lowercase letters correspond to the −21M13 forward sequencing primer) and ES8 (5′-caggaaaca gctatgaccCACTTCTCCAATTGTCCCTCA, 7667-7647, HIV-1 HXB2; the lowercase letters correspond to the M13 reverse sequencing primer), which amplify the V3-V5 region of *env*, resulting in a product of approximately 702 bp, of which approximately 626 bp are target dependent (17). HIV-1 proviral DNA quantitation was performed by duplicate serial endpoint dilution of infected PBMC DNA followed by nested PCR. The final concentration of $MgCl_2$ for all PCRs was 1.25 mM, and PCR was performed as follows: the first three cycles included incubations at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by an additional 32 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min in a Perkin Elmer 480 thermocycler. The last cycle of each round was followed by incubation for 5 min at 72°C.

cDNA synthesis and quantitation of plasma viral RNA. Plasma viral RNA load was determined by the Quantiplex HIV-RNA assay (bDNA assay; Chiron Corporation, Emeryville, Calif.) and by EPD of cDNA PCR products. For EPD, 200 μl of plasma was diluted to 1.2 ml with phosphate-buffered saline and ultracentrifuged at 45,000 rpm at 4°C for 1 h, followed by RNA extraction with RNAzol B (Biotecx, Houston, Tex.). The cDNA synthesis was performed by using primers ED12 and SuperScript II RNase H-free reverse transcriptase (Life Technologies, Gaithersburg, Md.) at 42°C for 1 h. Primers ED31 (5′-CCTCAG CCATTACACAGGCCTGTCCAAAG, 6816-6844, HIV-1 HXB2) (15) and ES8 were then used for the first round and ES7 and ES8 were used for the second round of PCR amplification. Serial endpoint dilution of cDNA and estimation of viral copy number were as described above.

Heteroduplex tracking assay. Probes were generated by subjecting ES7-ES8-derived PCR products to an additional three cycles of PCR in the presence of [α-^{33}P]dATP and 33 μmol of each deoxynucleoside triphosphate. Probes were then mixed with driver at the ratio of 1:100 in annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA; see legend to Fig. 2 for viral template copy numbers used in each reaction), denatured in boiling water for 2 min, and then immediately placed on ice for 10 min followed by heating to 55°C for 10 min to form heteroduplexes. The resulting reaction mixtures were electrophoresed in 5% neutral polyacrylamide gels at 250 V for 3 h (17), dried, and scanned by using a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager.

Molecular cloning and DNA sequencing and analysis. PCR products were cloned prior to DNA sequencing. The primers DR7 (5′-TCAACTCAACTGCT GTTAAATGGCAGTCTAGC, 6989-7020, HIV-1 HXB2) and DR8 (5′-CACT TCTCCAATTGTCCCTCATATCTCCTCC, 7667-7637, HIV-1 HXB2) were used in second-round PCRs, and the products were cloned into pCRII (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions. Clones bearing inserts were identified by illustrative restriction digestion and sequenced by using fluorescent-labeled M13 forward and reverse primers on an Applied Biosystems Inc. (Foster City, Calif.) 373 automated sequencer. Sequence editing and assembly was performed by using Sequencher, version 3.0 (Gene Codes Corporation, Inc., Ann Arbor, Mich.). All sequences were aligned by using the program PILEUP of the Genetics Computer Group Package (University of Wisconsin, Madison), version 8.1, and then edited manually by using the Genetic Data Environment (75). Neighbor joining, as implemented in the Phylogeny

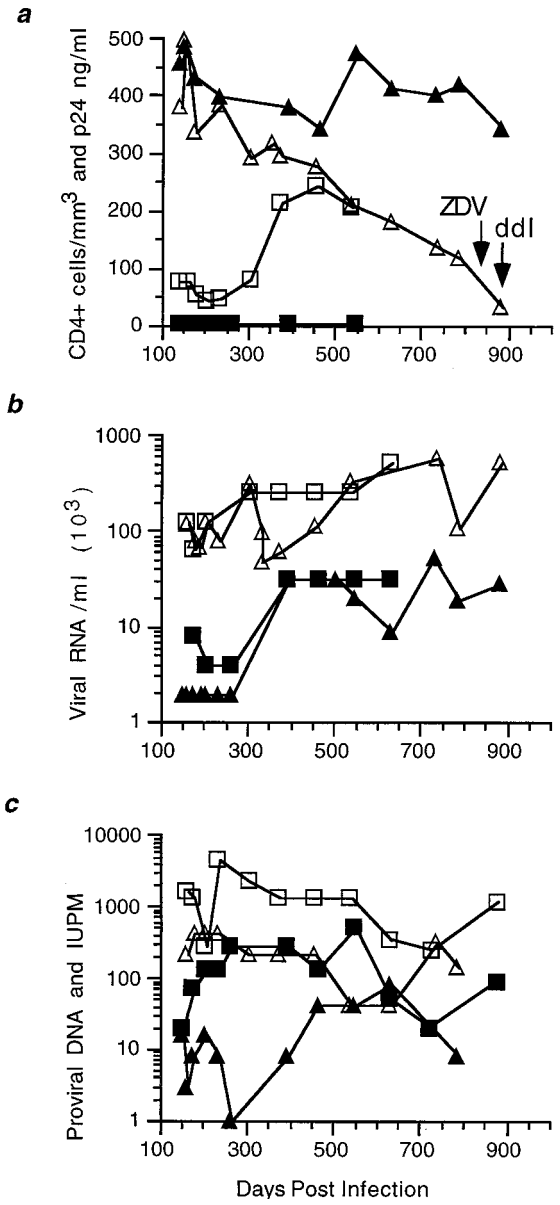

FIG. 1. Sequential analysis of peripheral blood $CD4^+$ T-cell counts and virus load in two HIV-1-infected patients. In each panel, filled symbols correspond to patient A and open symbols correspond to patient B. (a) $CD4^+$ T-cell counts (cells/μl) are shown with triangles, and p24 antigen levels (ng/ml) are shown with squares. (b) Viral RNA load in plasma (copies/ml) measured by using the bDNA assay shown with triangles and by EPD quantitation of *env* shown with squares. bDNA values plotted below 10×10^3 were negative by this assay. The viral RNA copy number in plasma, determined by both EPD and the standard bDNA method, provided generally comparable data. *env* fragment representation was found to be underestimated, however, if larger fragments (e.g., 1.2 kb) were assayed by EPD, especially if the samples were frequently thawed or stored at −80°C for more than 1 year (data not shown). (c) IUPM are shown with triangles, and proviral DNA per million cells in PBMC measured by EPD of *env* are shown with squares.

Inference package (PHYLIP), version 3.5c (J. Felsenstein, University of Washington, Seattle), was used to construct the phylogenetic tree, using two-parameter distance (35) with a ratio of transitions to transversions of 2:1. DNA sequence divergence was calculated by using the PHYLIP program DNADIST. The program MEGA (Molecular Evolution Genetic Analysis, University Park, Pa.) was used to estimate the average number of nonsynonymous (d_n) and synonymous (d_s) substitutions per nonsynonymous or synonymous site, respectively (43). All positions with an alignment gap in at least one sequence were excluded from pairwise comparison analysis.

Characterization of CCR5 genes and chemokine levels in plasma. PBMC DNA from days 635 and 887 PI from both patients was used as a template for PCR with the primers SF51 (5′-ATGCTGGTCATCCTCATCCTGATAAAC TGC, 199-219, GenBank accession number U54994) and SF52 (5′-TGGCAA TGTGCTTTTGGAAGAAGACTAA, 1006-979, GenBank accession number U54994) (66). PCR products were then electrophoresed on a 5% polyacrylamide gel to determine fragment length and then cloned into pCRII vector and sequenced by using the methods described above. Chemokine levels (RANTES, MIP-1a, MIP-1β) were measured in plasma by enzyme-linked immunoassay (ELISA) according to methods described by the manufacturer (R&D Systems, Minneapolis, Minn.).

Virus culture and neutralizing antibody. PBMC taken 152 days PI from patient A (common virus) and plasma taken 164 days PI from patient B (unique virus) were cocultured with PBMC taken from the same uninfected donor to generate a low-virus-passage stock, with titers determined at 1.3×10^5 and 7.7×10^4 50% tissue culture infective doses ($TCID_{50}$)/ml, respectively. Heteroduplex mobility and tracking assays confirmed the identity and homogeneity of each virus stock. HIV-1 SF2 was obtained from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, AIDS reagent repository and grown in donor PBMC, and a supernatant stock of 5×10^5 $TCID_{50}$ stored at −195°C was used for all assays.

Neutralizing antibody titers were determined against the lab strain SF2 as well as both common and unique variants. For SF2, positive-control HIV-immune sera and six fourfold dilutions of patient sera from 1:10 to 1:10,240 were incubated with 5 and 10 $TCID_{50}$ for 1 h at room temperature. The virus serum mixture was added to 5×10^5 PBMC previously stimulated with phytohemagglutinin (PHA) for 72 h, and cultures were assayed for viral p24 by ELISA (Abbott Laboratories, Chicago, Ill.) after 10 days. For the common and unique variants, positive-control HIV-immune sera as well as serial fourfold dilutions of patient A and B plasma from 1:20 to 1:1,280 were inoculated with six fourfold dilutions of virus ranging from 576 to 0.56 $TCID_{50}$ for patient A and 224 to 0.22 for patient B $TCID_{50}$ for 1 h at room temperature. The virus-serum mixture was then inoculated onto 2×10^5 PBMC previously stimulated with αCD3/αCD28 (0.1 μg/ml) and incubated for 7 days for microtiter reverse transcriptase (RT) assay. Cultures were washed carefully to remove sera before the measurement of p24 and RT. HIV neutralization titers were calculated as 90% reduction in titer compared to HIV antibody-negative serum. All serum and virus dilutions were done in duplicate and run in parallel, and the same donor PBMC was used for all neutralization studies and for generating all virus stocks. The neutralization titers of HIV immunoglobulin against HIV-1 SF2, the common variant (patient A), and the unique variant (patient B) were 1,050, 20, and 60, respectively.

Lymphoproliferative responses. Lymphoproliferative responses were performed as previously described (49). Briefly, PBMC were incubated in quadruplicate replicate culture at 10^5 cells per well in 96-well round-bottomed plates for 6 days in the presence of either PHA (1 μg/ml), *Candida* (1 μg/ml), HIV-1 SF2 gp120 (10 μg/ml), or HIV-1 SF2 p24 (1 μg/ml). On day 6, wells were pulsed with 2.5 μCi of [^{3}H]thymidine for 18 h and then harvested. Responses are expressed as a stimulation index (SI), which is calculated from the mean of quadruplicate counts-per-minute measurements of ^{3}H-thymidine incorporation of PBMC stimulated with antigen, divided by the mean of incorporation into PBMC in the absence of antigen stimulation.

Precursor CTL (pCTL) frequencies. pCTL frequencies were determined by limiting dilution analysis. Briefly, 24 replicates of fresh PBMC were plated in 96-well round-bottomed plates at serially diluted cell concentrations ranging from 6,250 to 200,000 cells/well with 500 autologous gamma-irradiated (3,000 rads) rVV-EGP (68)-infected monocytes per well (as stimulator cells) and 5×10^4 autologous gamma-irradiated PBMC feeders in RPMI containing 10% human AB serum and 10 U of human recombinant interleukin-2 per ml. On day 10, effectors were tested in a 4-h ^{51}Cr release assay with target cells, 5×10^3 autologous Epstein-Barr virus-transformed B-lymphoblastoid cells, previously infected with recombinant vaccinia viruses containing either HIV-1LAI *env* (vPE-16) (21), HIV-1LAI *gag* (vDK-1) (50), HIV-1LAI *pol* (vRT) (23), or the control *lacZ* gene (vSC-8) (5), and labeled with radioactive ^{51}Cr. Chromium release from 16 replicates of target cells incubated with media alone (referred to as spontaneous release) was always less than 20% of the chromium release of target cells incubated with 5% Triton-X (referred to as maximum release). Wells with lysis exceeding the mean spontaneous release by three standard deviations were considered positive. pCTL frequency was estimated from the initial cell number at which 37% of the wells were negative. Values were determined by the chi-square minimization method (6). pCTL against the control targets expressing *lacZ* were <2 per 10^6 in all experiments, and HIV-1-specific CTL were <0.1 per 10^6 PBMC in five HIV-1-seronegative subjects.

Statistical analyses. Paired Student's *t* test was used to compare certain parameters between the two patients, including rates of synonymous and nonsynonymous substitutions (with standard errors given automatically by the MEGA program), lymphoproliferative responses, and pCTL frequencies.

Nucleotide sequence accession numbers. Viral sequences can be retrieved from GenBank, using accession no. U56146-U56235 and U79034-U79113.

RESULTS

Viral load in PBMC and plasma. Viral load is currently the best predictor of disease progression (51). Furthermore, for analysis of quasispecies diversity and turnover, it was necessary to determine the number of template molecules of virus examined in each experiment (45). We therefore quantitated proviral DNA and viral RNA copy numbers by using EPD followed by nested PCR of the same 0.7-kb *env* gene fragments (spanning the third through fifth variable regions of the gp120 coding sequence) used in subsequent heteroduplex and DNA sequencing experiments.

Plasma RNA viral load was quantified in all samples by using the Chiron bDNA assay and in a subset by EPD. The two methods provided comparable results (Fig. 1b), with viral load in patient A consistently between $<1 \times 10^4$ and 5×10^4 copies/ml, compared to 1×10^5 and 6×10^5 copies/ml in patient B (Fig. 1b). PBMC-associated proviral DNA was quantified by EPD only (Fig. 1c). Proviral load in patient A increased until 265 days PI and then fluctuated around that level thereafter. Patient B had a higher initial viral load and, as with viral RNA, generally maintained a 10-fold higher level throughout the study period. Similarly, viral load measured by culturable virus in patient B was usually about 10-fold higher than in patient A (Fig. 1c).

Same source of HIV-1 infection evaluated virologically. We sought to determine if epidemiological evidence for a common source of infection of the two patients (see Materials and Methods) could be confirmed virologically. We conducted heteroduplex tracking assays (HTA) (15) using as probes uncloned (data not shown) and cloned viral DNA derived from PBMC from the initial time point examined from each patient and, as a driver, PCR products amplified from all available time points of the PBMC, plasma, and virus isolates from each patient. In the HTA, the electrophoretic mobilities of heteroduplexes are proportional to the number of mismatched and unpaired bases between annealed strands (17). HIV evolves at such a high rate in vivo that this analysis can be used to detect epidemiologically linked infections as well as distinguish between variants present at different times in the same infection (16, 17). By using a patient A probe, nearly identical virus, indicated by heteroduplexes comigrating with homoduplexes (homo- and heteroduplexes produced from the reannealed probe alone are in lanes marked P), was detected in PBMC and plasma at the initial time point examined in both patients (Fig. 2a and c), with the virus populations then diversifying over time in each patient. This supports a common source of infection for the two men.

Two variant populations were evident in patient B, the one referred to above detected as fast-migrating heteroduplexes with the patient A probe, and the second migrating with a mobility of approximately 0.6 relative to the homoduplexes (Fig. 2c). The patient B probe employed corresponded to this second variant virus population (Fig. 2d) and was not found in patient A (Fig. 2b). This suggested the possibility of infection of patient B with two highly divergent variants from the original source partner or of a second infection from another source. Unfortunately, although the source contact for the two patients was willing to confirm the clinical and epidemiologic aspects of the sexual contact with patients A and B, he refused

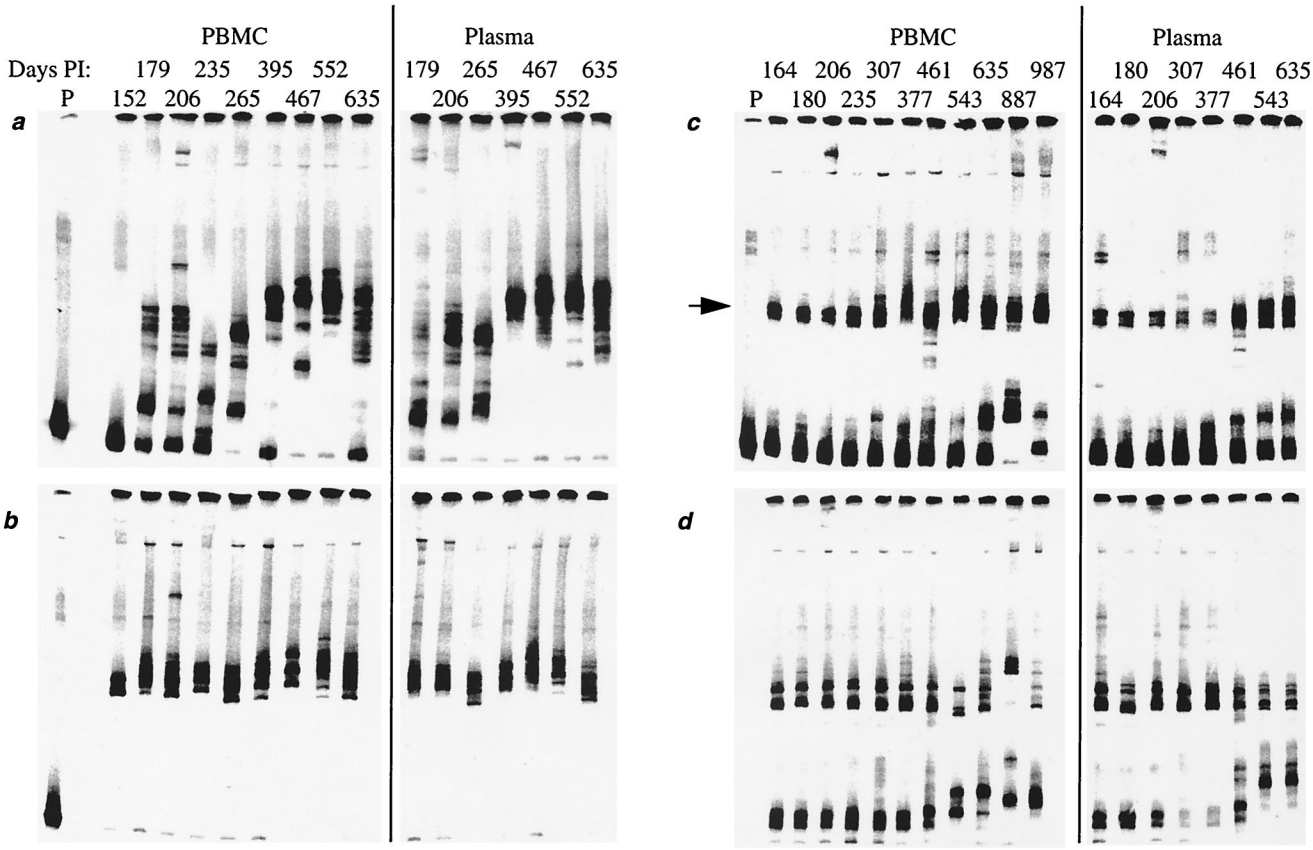

FIG. 2. Viral quasispecies representation and turnover in vivo displayed by HTA. The probe employed in panels a and c was a PCR fragment amplified from a molecular clone derived from the PBMC from the initial, day 152 time point from patient A. The probe employed in panels b and d corresponded to the unique variant and was similarly derived and taken from the day 164 PBMC from patient B. Driver sequences were from patient A in panels a and b and from patient B in c and d. The arrow in panel c indicates the unique variants found in patient B. For all plasma samples, RT-PCR products amplified from approximately 100 copies of HIV-1 RNA templates were used as driver. For PBMC samples, PCR products amplified from about 50 copies of HIV-1 genomic DNA were used as driver except samples for days 152 and 635 from patient A and day 206 from patient B, all of which contained roughly 20 copies of template genomic DNA.

sampling for molecular virologic analysis. Detailed sexual history interviews of each patient taken separately did not suggest any other source of infection between the time of sexual contact with the source partner and entry into this study. Thus clinically, it appears that infection with the unique variant arose from the same encounter.

Patterns of virus evolution detected by HTA. To evaluate the virologic changes that occurred during the course of infection in these two patients, we characterized HIV-1 genome fragments following PCR from sequential PBMC and plasma (Fig. 2) as well as PBMC- and plasma-derived virus isolates (data not shown) taken 152 to 987 days PI. HTA analysis of the viral *env* quasispecies developed in the PBMC of patient A reveals a relatively homogeneous virus population at 152 days PI, which then diversified greatly by day 179 PI and stayed high thereafter (Fig. 2a). Virus highly similar or homologous to the original viral species replicating in patient A, revealed by bands comigrating with the homoduplexes in Fig. 2a, was only infrequently detected after day 235, around the time of a 10-fold increase in plasma viral RNA load. In patient B, both the common and unique virus populations evident at 164 days PI diversified only slightly from day 307 onward in the PBMC (Fig. 2c and d), around the time of a transient eightfold drop in RNA virus load measured by the bDNA but not the EPD assay. Virus homologous to the initial viral species common to both patients was detected in patient B throughout 635 days of follow-up, whereas virus highly homologous (comigrating with the homoduplexes) to the viral species unique to patient B was detected through day 461. These data indicate that the virus populations are evolving and/or being replaced much quicker in the slower progressor (patient A), than in the fast progressor (patient B).

The evolution of plasma virus populations appeared to be ahead of that found in PBMC, at least in patient A, consistent with previous studies evaluating the appearance of drug resistance mutations in these two compartments (78). For example, the initial variant of patient A continued to be detected in the PBMC at day 235, while in plasma it was substantially lost before day 206 (Fig. 2a). Similarly, virus populations in the PBMC-derived virus isolates changed ahead of that found in the PBMC (data not shown), consistent with a previous report (16). However, no such differences in the loss of appearance of variant populations were detected in the more slowly evolving variant populations within patient B.

DNA sequence analysis. For a more detailed analysis of HIV-1 evolution in these two patients, the fragments of the viral genome evaluated by HTA (V3-V5 region of *env*) from the PBMC at four time points and the plasma at the 635-day time point were subjected to molecular cloning and DNA sequence analysis. Seven to nineteen clones were analyzed from each sample. Based on the number of input template molecules into the PCRs used to derive these clones, we expect

TABLE 1. Nucleic acid sequence analyses in patients A and B

Days PI	No. of clones sequenced	No. of templates sampled[a]	Mean divergence at days PI[b]:					d_n (SE)	d_s (SE)	d_n/d_s[c]	P[d]
			152/164	179/180	635	635-Pl	887				
Patient A											
152	10	20	0.42					0.0038 (0.0013)	0.0110 (0.0042)	0.36	<0.2
179	10	50	2.47	1.91				0.0302 (0.0046)	0.0188 (0.0071)	1.61	<0.2
635	10	20	4.19	4.12	2.28			0.0389 (0.0055)	0.0209 (0.0083)	1.86	<0.1
635-Pl	10	100	5.52	4.31	3.35	2.88		0.0271 (0.0040)	0.0249 (0.0071)	1.09	>0.5
887	14	50	7.14	5.93	5.60	5.04	2.89	0.0333 (0.0044)	0.0358 (0.0081)	0.93	>0.5
Patient B[e]											
Common variants											
164	10	50	0.72					0.0087 (0.0024)	0.0059 (0.0036)	1.47	>0.5
180	10	50	1.00	0.93				0.0082 (0.0023)	0.0192 (0.0067)	0.43	<0.2
635	10	50	1.88	2.13	1.51			0.0144 (0.0030)	0.0236 (0.0076)	0.61	<0.5
635-Pl	7	100	2.25	2.77	2.04	1.68		0.0123 (0.0030)	0.0259 (0.0084)	0.47	<0.2
887	11	50	2.08	2.25	2.11	2.63	1.55	0.0075 (0.0023)	0.0523 (0.0114)	0.14	<0.005
Unique variants											
164	10	50	0.56					0.0047 (0.0018)	0.0115 (0.0053)	0.41	<0.5
180	19	50	0.87	0.84				0.0070 (0.0019)	0.0160 (0.0057)	0.44	<0.2
635	10	50	1.41	1.47	0.65			0.0045 (0.0016)	0.0148 (0.0051)	0.30	<0.1
635-Pl	7	100	1.50	1.57	0.93	1.02		0.0088 (0.0022)	0.0254 (0.0085)	0.35	<0.1

[a] All sequences were derived from proviral DNA except those on day 635, at which time plasma viral sequences (635-Pl) were also determined. This estimate was obtained from the viral genome copy number in the material used for PCR and cloning (45).
[b] A matrix providing the average divergence between all pairwise comparisons of molecules from within each time point or between time points.
[c] This ratio provides an indication of the relative selective pressures on the viral population (see text).
[d] Probability that the difference between d_n and d_s is random.
[e] For patient B, the mean divergences between common and unique variants were as follows: day 164 PI, 15.4; day 180 PI, 15.6; day 635 PI, 15.6; day 635-Pl PI, 15.7.

that most or all of the clones examined were derived from a unique viral genome template (Table 1) (45). As with the HTA studies, two variant populations were present in patient B, one of which, the "common" form, was genetically indistinguishable from that found in patient A at the initial time point examined (mean 1.1% divergence between the two patient virus populations, compared to intrapatient divergence levels of 0.42 and 0.72% for patients A and B, respectively) (Table 1), further substantiating the same source of infection. The two variant populations in patient B were an average of 15.5% divergent throughout the period of study, as expected for epidemiologically unlinked infections (41). It should be noted, however, that infection with multiple distinct viruses has also been reported (84).

Calculation of virus population diversities based on DNA sequence analysis paralleled that demonstrated by HTA (Table 1). In patient A, intrasample proviral diversity rose dramatically between the 152- and 179-day time points (0.42 to 1.91%) and increased gradually thereafter to 2.89% at 887 days PI. Intersample comparisons revealed a pattern of rapid provirus population turnover; the divergence compared to the initial time point grew to 2.47% at 179 days and to 7.14% by day 887, an average increase of 2.74% per year (Table 1 and Fig. 3a). In marked contrast, both provirus populations in patient B were more homogeneous at successive time points and turned over much more slowly. The common-form provirus populations only doubled in complexity between 164 and 887 days (0.72 to 1.55%), and these two populations differed from one another by only 2.08% (Table 1 and Fig. 3a). The unique provirus population complexity grew and diverged from the initial population at virtually the same rate as the common form, although the 887-day time point was not evaluated (Table 1 and Fig. 3a). The rates of divergence noted for the common and unique populations in patient B were 0.66 and 0.55% per year, respectively. Each plasma viral RNA population examined at 635 days PI was more complex than the contemporary PBMC proviral population, as well as more divergent from the PBMC DNA at the initial time point (Table 1 and Fig. 3a).

Similar patterns of evolution were evident from phylogenetic analysis (Fig. 4). Beyond the first time point, shorter branch lengths (less divergence) were noted for viruses evolving in patient B compared to those in patient A. The two virus populations evident in patient B are also shown to be phylogenetically unlinked. Displayed along with the patient A and B sequences are the most closely related sequences taken from the HIV sequence database. As can be seen, there is no tendency for the patient B common and unique virus populations to cluster together, again, consistent with infection of this patient from more than one source or with multiple sources of infection in the known source and then transmission of both strains to only patient B.

Single viral sequences taken from the plasma and PBMC from patient B 635 days PI were found to be recombinants between the common and unique forms of virus, as were multiple proviral sequences from the 887-day time point (data not shown). We were unable, however, to detect similar recombinants when using the 635-day PBMC recombinant sequence as a probe in HTA (data not shown), possibly due to its minor representation in the virus mixture. It is formally possible, however, that these recombinants did not form in vivo but were derived by template switching during PCR (52). These sequences are not reported in the analyses shown in Table 1 and Fig. 4.

Virus phenotype. Analysis of viral amino acid sequences derived from both patients failed to reveal any of the arginine or lysine residues in the V3 region previously associated with syncytium induction and rapid disease progression (13, 14, 24). However, each unique variant sequenced at 635 days PI in patient B did contain an arginine-to-serine substitution in the

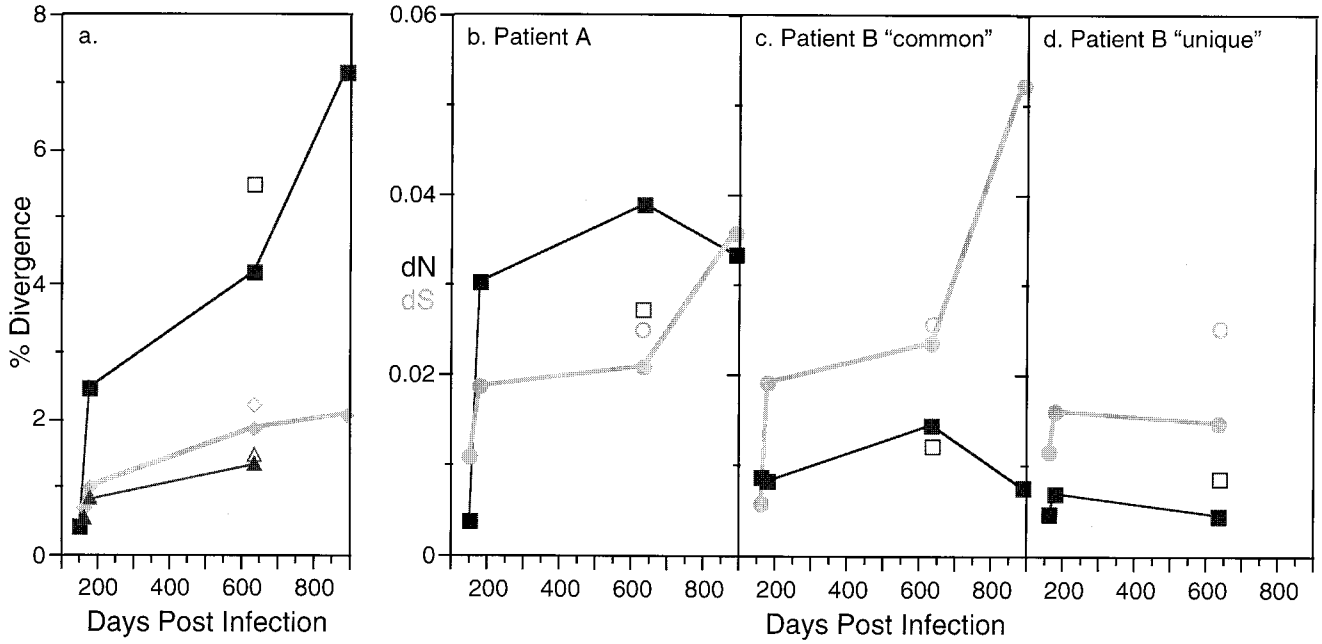

FIG. 3. Divergence of viral sequences over time in patients A and B. (a) The average degree of divergence of viral sequences from those at the initial time point is plotted for the PBMC proviruses from patient A (■) and the patient B common (◆) and unique (▲) variants. The divergences of plasma viral sequences at 635 days versus the earliest time point of PBMC-associated proviral sequences in each patient are shown (□, patient A; ◇, patient B). (b to d) d_n (squares) and d_s (circles) for each virus population. PBMC populations are shown with filled or shaded symbols over time and plasma populations with open symbols at the 635-day time point.

GPGR(S) motif at the crown of the V3 loop, a motif believed to be responsible for inducing a β-turn in the protein and critical to recognition by some neutralizing antibodies (44). Almost every clone of common virus examined at 635 and 887 days PI in patient A had a threonine-to-alanine change at position 22 of the V3 loop. This is the most common change in uncharged amino acids in this region (54). Virus isolates were generated from both patients at nearly all time points. Each replicated with equal efficiency in primary cultures of normal blood donor cells and monocyte-derived macrophages and, as predicted by the V3 loop sequence analysis (40), did not induce syncytia in the MT-2 cell line.

Accumulation rates of synonymous and nonsynonymous substitutions. Analysis of the types of mutational changes that occur in virus populations provides insight into the selective forces acting on these populations. The proportion of synonymous codon sites that have changed (d_s) is largely a function of the number of replication cycles the population has undergone, except in regions critical to RNA structure or translation of multiple reading frames. If d_s is high relative to the number of nonsynonymous codon sites that have changed (d_n), this suggests that purifying selection for replication fitness is predominating, a bias routinely observed for cellular genes (36). Increasing levels of d_n relative to d_s suggests that mutations are accumulating more randomly and that diversifying selective pressures by host factors such as an immune response are selecting for escape variants (22, 31, 32). d_n/d_s ratios of >1 have been noted in several analyses of HIV. This has been interpreted to indicate a predominance of positive (or diversifying) pressure on the virus population imposed by the immune system (1, 2, 29, 47, 73, 74, 80).

To assess the selective forces associated with *env* diversification in these two patients, we determined d_s and d_n in each provirus and plasma viral population sequenced (Table 1 and Fig. 3). In patient A, a bias for nonsynonymous site changes was found in the 179- and 635-day time points with a d_s bias at day 152 and near-equivalent rates of d_n and d_s at 887 days. A bias for synonymous site changes was consistently found in both provirus populations in patient B, except for the common-form variants at the earliest time point. Most of the intrasample biases noted, however, were not statistically significant at the 95% confidence level, although several P values were <0.1 (Table 1). The lone exception was the common variant provirus population in patient B at 887 days PI, in which d_s was significantly higher than d_n ($P < 0.005$). It should be noted that previous measures that have revealed d_n biases have also failed to reach statistical significance (69). In both patients, a greater bias for d_s selection was found in the plasma relative to the PBMC-derived virus at the same time point (Table 1 and Fig. 3).

Characterization of CCR5 genes and chemokine expression. Heterozygosity for a functional CCR5 HIV1 coreceptor gene has recently been associated with relatively somewhat slower disease progression (12, 30). To attempt to rule out this as an explanation for slower disease progression in patient A, we applied PCR to amplify an 808-bp product (77% of the coding sequence) spanning the 32-bp deletion shown to be responsible for inactivation. PCR products from both patients were examined on 5% polyacrylamide gels and were found to be wild type in length (data not shown). Furthermore, sequence analysis of these products revealed that 9 of 10 clones (5 from each patient) were 100% homologous with wild-type CCR5 (66), whereas one sequence contained a single synonymous site mutation (data not shown). Thus, no evidence of CCR5 heterozygosity was associated with the divergent disease progression in these two patients. Furthermore, similar levels of the chemokines RANTES, MIP-1a, and MIP-1β were produced in the plasma of both patients sampled between 461 and 635 days PI (data not shown).

Virus-specific immune responses. To examine the host selective forces that may be driving the observed viral evolution in the two patients, we conducted assays to detect virus neutralizing antibody, HIV-specific pCTL frequencies, and lym-

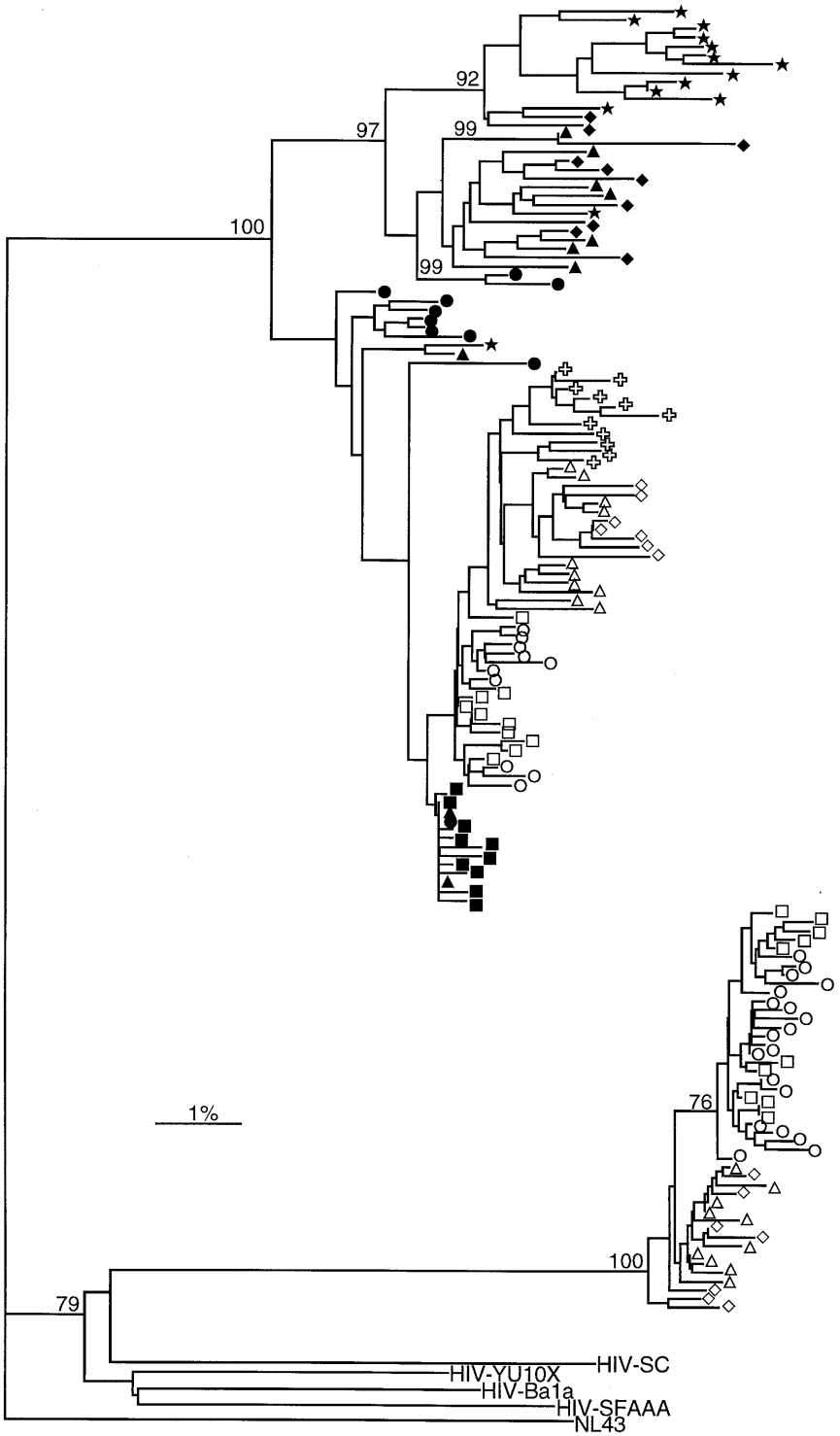

FIG. 4. Phylogenetic analysis of HIV-1 *env* evolution in patients A and B. Filled and open symbols correspond to patients A and B, respectively. Viral sequences derived from PBMC at 152 and 164 days PI, respectively, for patients A and B are shown with squares and at 179 days (patient A) or 180 days (patient B) are shown with circles. PBMC sequences from 635 days PI are shown as triangles, and plasma sequences are shown as diamonds. PBMC-derived sequences from patient A at 887 days are indicated by filled stars and from the common variant in patient B are indicated by open crosses. The numbers at branch points indicate groupings found in at least 75 of 100 neighbor joining bootstrap repetitions. Prototype HIV-1 *env* subtype B strains NL43 (GenBank accession number M19921), HIV-SC (M17459), HIV-YU10X (M93259), HIV-Ba1a (M63929), and HIV-SFAAA (M65024) were used as outgroups. Horizontal length equivalent to 1% divergence is shown.

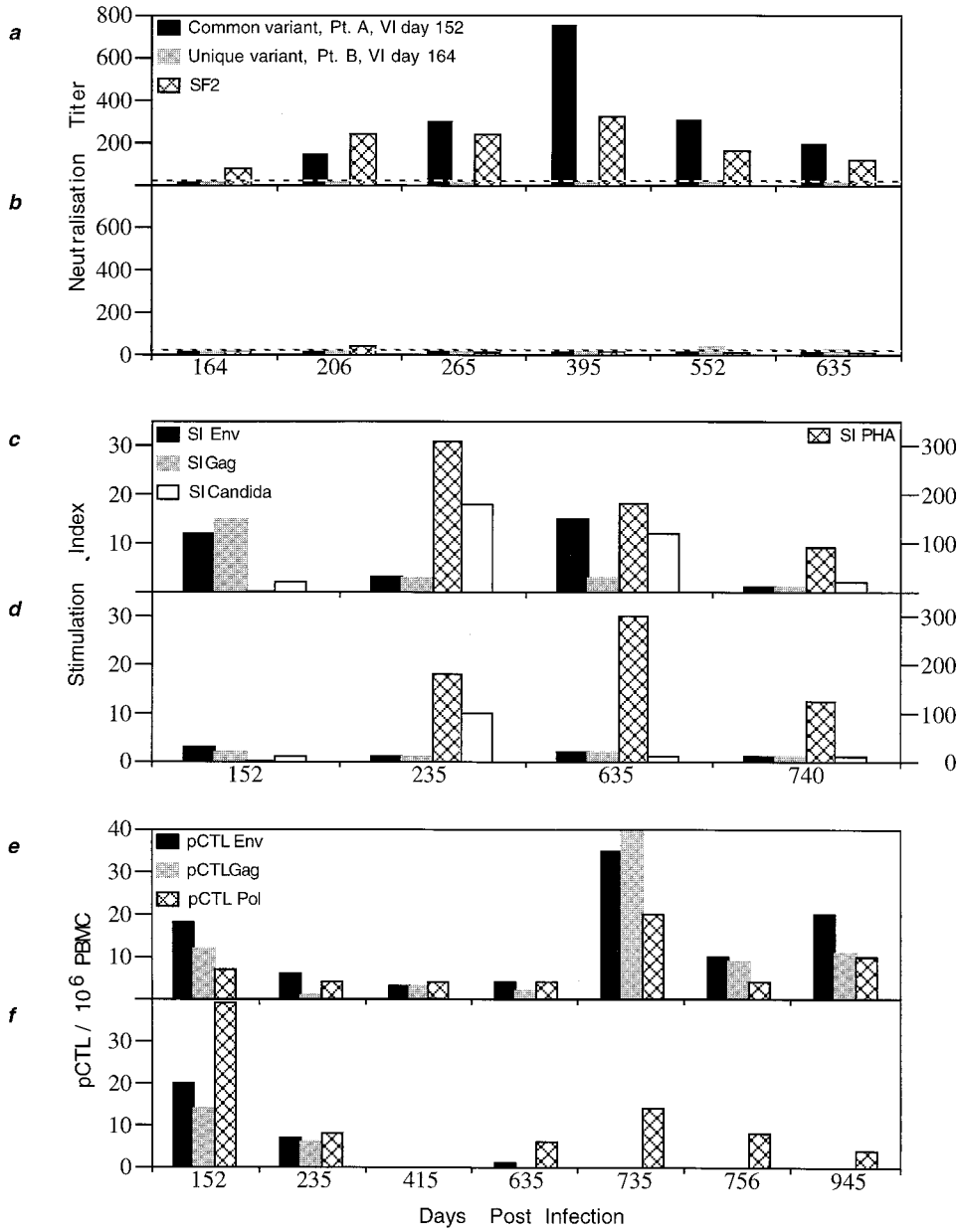

FIG. 5. Neutralizing antibody titers against autologous (patient A and patient B isolates from the earliest time points) and heterologous (lab strain HIV SF2) viruses are shown for patients A (a) and B (b). The broken line along the bottom of the graph indicates a titer of ≤1:10, the lowest dilution of sera. Lymphoproliferative responses measured as SI to PHA, *Candida*, HIV-1 gp120, and HIV-1 p24 antigen in patients A (c) and B (d). The scale for the Env, Gag, and *Candida* responses is shown to the left of the panels, and the PHA response scale is shown to the right of the panels. pCTL frequencies (per million PBMC) recognizing autologous target cells expressing HIV-1 Env, Gag, and Pol in patients A (e) and B (f).

phoproliferative responses to viral antigens, using sequential specimens from each patient.

Sera taken from both patients were tested for neutralizing antibody against the laboratory strain HIV1 SF2 and virus isolates corresponding to the common variant isolated from patient A at 152 days PI and the unique variant isolated from patient B at 164 days PI. The genetic identity of each patient-derived virus isolate was confirmed by HTA. Sera taken from patient A throughout the period of study were found to have neutralizing antibodies against SF2 (Fig. 5a) as well as the common variant, with both titers peaking around 395 days of infection. In contrast, patient A sera did not neutralize the unique variant virus isolate from patient B (Fig. 5a). Sera from patient B demonstrated little or no neutralizing antibody against any of the three virus isolates (Fig. 5b). Hence, little humoral immune pressure against HIV could be demonstrated in the fast progressor.

To evaluate the T-cell memory responses in each patient, proliferation of PBMC was measured following in vitro stimulation with mitogen (PHA), recall antigen (*Candida*), and HIV-1 antigens (p24 CA and gp120 SU). As shown in Fig. 5c and d, both patients demonstrated depressed lymphoproliferative responses to PHA and *Candida* at the earliest time point, 152 days PI. This is consistent with our findings in a larger

cohort of individuals with recent HIV infection (58a). By 235 days PI, responses were transiently restored in both individuals, although lymphoproliferation in response to the recall antigen *Candida* was largely lost by 635 days PI in patient B and by 740 days PI in patient A. Distinct differences (though not statistically significant; $P < 0.1$) were noted in the two patients with respect to lymphoproliferation to HIV-1 antigens p24 and gp120 (Fig. 5c and d). Patient A developed one of the strongest HIV-specific T helper responses of the subjects with recent HIV infection analyzed (58a). Lymphoproliferation was detected in response to both p24 and gp120 at 152 days PI with SIs of 15 and 12, respectively, and the response to gp120 was again noted at 635 days PI with an SI of 15. In contrast, patient B failed to induce a HIV-specific T helper response as measured by lymphoproliferation, with SIs ≤ 3 for both HIV-1 antigens at all time points examined (Fig. 5d). These results indicated that although T-cell memory responses were impaired overall in both patients, only patient A mounted these responses to HIV-1 antigens over the course of infection ($P > 0.2$ for PHA and < 0.1 for *Candida* and the viral antigens CA and SU).

CTLs are believed to play a critical role in the control of viral infection. To determine the ability of these subjects to mount HIV-specific CTL responses, precursor CTL recognizing autologous target cells expressing HIV-1 Env, Gag, or Pol were measured in a limiting dilution assay from PBMC obtained at seven time points over the course of the study. As shown in Fig. 5e and f, both individuals had Env-, Gag-, and Pol-specific CTL responses at the earliest time point, 152 days PI. However, beyond 235 days of infection there were clear differences in the cytolytic profiles of the two individuals. Patient A maintained a broad CTL response, demonstrating CTL specific for HIV-1 Env and Pol at all seven time points and for HIV-1 Gag at five time points. Patient B, in contrast, had no detectable HIV-specific CTL at 415 days PI, and at subsequent time points responses were restricted to HIV-1 Pol-expressing target cells. These results indicated that over the course of infection, the breadth of CTL response was greater in patient A than in patient B ($P < 0.05$ beyond 235 days PI for the comparison of pCTL against Env versus < 0.1 for the entire period of analysis, and $P < 0.2$ and > 0.2 over both periods against Gag and Pol, respectively).

DISCUSSION

The foregoing analysis provides a detailed view of the evolution of virus and antiviral host immune responses in two patients infected near-simultaneously from the same source, yet one progressed to disease extremely rapidly while the other has experienced a more average course. Correlative evidence that host immune responses played a role in the differential rate of progression was found: more vigorous neutralizing antibody and lymphocyte proliferation responses correlated with slower disease progression, a finding consistent with a previous study by Schwartz et al. (72). The slower progressor in our study also had higher precursor CTL frequencies against HIV-1 Gag and Env (not Pol), but only after 235 days of infection. In a larger cohort of patients studied shortly after being infected with HIV-1, we found that pCTL frequencies (pCTL directed against at least one HIV gene product was detected in nearly all patients), viral load, as measured by plasma RNA levels, and $CD4^+$ T-cell counts all followed similar patterns during the first 18 months of infection (58a). Values were the highest at the very earliest time after infection and then declined over the ensuing 200 to 300 days and stabilized thereafter (58a). Responses appeared to differ between patients after the first year and were highly correlated with viral burden and disease progression (58a). Higher CTL levels have been associated with slower disease progression by other groups in both cross-sectional (68) and longitudinal (38, 53, 80) studies. Our results are consistent with the study by Klein et al. (38), who showed that Gag pCTL remained high in long-term asymptomatics but dropped off to undetectable levels in four of six rapid progressors (one other rapid progressor never had detectable Gag pCTL, and one retained high levels). However, the time of maximum pCTL levels relative to infection was variable in the Wolinsky et al. (80) study of six unlinked individuals, and in their study pCTLs against Pol were no less likely than against Gag or Env to distinguish the fast from the slow progressors. Thus, analysis of both precursor CTL frequencies, as shown here and as cited above, as well as bulk memory CTL in the recent study of Rinaldo et al. (67), have failed to demonstrate a fully consistent benefit of CTL in slowing disease course. Detailed analysis of epitope recognition is likely to be required to elucidate a protective role, if any, of CTL in HIV pathogenesis.

The rate of disease progression in patients A and B was reflected in the provirus and RNA viral load, as well as in the evolutionary trends observed in the provirus population. Viral RNA levels were 10-fold higher in the fast progressor than in the slow progressor. Mellors et al. (51) have shown that even three- to fivefold differences in plasma RNA levels are associated with faster disease progression. Based on the Mellors data, patient B would have been in the quartile of patients with the highest viral load, >50% of which progressed to AIDS in under 4 years. Virus load in patient A increased from <10,000 in the first year to the 10,000 to 40,000 range in the following two years, levels that traverse the uppermost three quartiles of patients defined by Mellors. Virus load measured by IUPM and proviral DNA also showed a generally consistent log higher abundance in the fast progressor. By comparing these latter two measures, we found that the relative number of infectious units derived from PBMC per PBMC provirus varied around the ratio 1:10 for both patients.

The finding of the second variant virus population in the fast progressor complicates our analysis of the relative contribution of host responses versus virus genotype in determining the rate of disease progression, while providing a unique opportunity to study the evolutionary patterns of two virus populations in the same host. Despite repeated denials of other sexual contacts to account for the unique variant in patient B, we found no evidence of this virus having been passed to patient A, including no functional humoral immune recognition of this virus in patient A.

The provirus population in the slower progressor, patient A, diversified in a nonlinear fashion, as can be seen from both the heteroduplex and DNA sequence data, with a tremendous change occurring during the 152- to 179-day interval. Overall, the proviral DNA population in this slower progressor diverged much more rapidly than that in the rapid progressor, patient B. In patient A, the *env* fragment population at 887 days was on average 7.14% divergent from the population 735 days earlier. This amounts to a rate of 2.74%/year over this interval, or 2.9%/year from the point of infection (assuming that the population was initially homogeneous). In contrast, the corresponding variant proviral population in patient B diverged from the proviral population at the initial time point by only 2.08% over the same interval (0.66%/year; extrapolated to 0.86%/year from the start of infection). Similarly, the unique variant proviral population in patient B diverged by only 1.41% over the 471-day interval assessed (0.55%/year). The similar diversification rate in these two variant populations

in patient B might suggest that the increased viral evolutionary rate in patient A is due to host factors. However, it is also possible that the unique variant disabled the critical host responses early in infection such that it became incapable of driving the evolution of either viral strain.

Analysis of synonymous and nonsynonymous site mutations revealed weak, sporadic evidence for a growing diversifying selection in the proviral population in patient A, the slower progressor, as evidenced by a preponderance of d_n versus d_s mutations in the *env* fragment analyzed. In contrast, both variant proviral populations in the fast progressor (patient B) reflected a purifying selection expected for populations not subjected to significant immunological pressure. In all cases, the d_n/d_s ratio was lower in plasma virus versus concurrent provirus populations, possibly reflecting a trend towards selection of more rapidly replicating viruses in this more dynamic virus compartment.

These results, along with those of Lukashov (47) and Wolinsky (80), confirm and extend our previous cross-sectional analysis of viral diversity in fast versus slow progressors and strengthen our hypothesis that individuals capable of mounting a strong immune response against the virus drive the successive evolution of multiple virus variants, while those individuals with rapid disease progression fail to substantially drive this evolution (16). Thus, as shown previously and reiterated here, increased proviral genetic diversity correlates with slower disease progression (16, 46, 47, 80).

Since the fast progressor failed to sustain a detectably substantial immune response to either of his two divergent virus populations, it is not clear whether his ability to respond was adversely affected by the virulent effects of the unique variant or whether he had an inherent inability to maintain a response to most HIV strains. Furthermore, the two independently evolving virus populations in the fast progressor were not found to have achieved an appreciable level of genetic or thereby, antigenic diversity. These data are not consistent with a strict interpretation of the antigenic threshold hypothesis (60), which holds that accumulation of immunologically distinct virus variants is required for disease induction, eventually exhausting the capacity of the immune system to respond and leading to the development of AIDS in infected individuals. In the case of fast progressors, however, it has been argued that a critical threshold of viral diversity is achieved, but that this level is quite low (59). However, that antigenic thresholds correspond to critical junctures in the progression to AIDS remains to be established.

The correlates of disease progression revealed in this study were established both early (virus load) and later (when evolutionary and immunological response trends were established) in infection. Detailed evaluation of the virological and host responses of common source infections thus provides an important approach for analysis of HIV disease progression.

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