Changes in Human Immunodeficiency Virus Type 1 Fitness and Genetic Diversity during Disease Progression†

Ryan M. Troyer,¹ Kalonji R. Collins,² Awet Abraha,¹ Erika Fraundorf,¹ Dawn M. Moore,¹
Randall W. Krizan,¹ Zahra Toossi,¹ Robert L. Colebunders,³ Mark A. Jensen,⁴
James I. Mullins,⁴ Guido Vanham,³ and Eric J. Arts^{1,2*}

Division of Infectious Diseases, Department of Medicine,¹ and Molecular Virology Program,² Case Western Reserve University, Cleveland, Ohio 44106; Laboratory of Immunology, Institute of Tropical Medicine, Antwerp, Belgium³; and Department of Microbiology, University of Washington, Seattle, Washington⁴

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This study examined the relationship between ex vivo human immunodeficiency virus type 1 (HIV-1) fitness and viral genetic diversity during the course of HIV-1 disease. Primary HIV-1 isolates from 10 patients at different time points were competed against control HIV-1 strains in peripheral blood mononuclear cell (PBMC) cultures to determine relative fitness values. Patient HIV-1 isolates sequentially gained fitness during disease at a significant rate that directly correlated with viral load and HIV-1 *env* C2V3 diversity. A loss in both fitness and viral diversity was observed upon the initiation of antiretroviral therapy. A possible relationship between genotype and phenotype (virus replication efficiency) is supported by the parallel increases in ex vivo fitness and viral diversity during disease, of which the correlation is largely based on specific V3 sequences. Syncytium-inducing, CXCR4-tropic HIV-1 isolates did have higher relative fitness values than non-syncytium-inducing, CCR5-tropic HIV-1 isolates, as determined by dual virus competitions in PBMC, but increases in fitness during disease were not solely powered by a gradual switch in coreceptor usage. These data provide in vivo evidence that increasing HIV-1 replication efficiency may be related to a concomitant increase in HIV-1 diversity, which in turn may be a determining factor in disease progression.

Human immunodeficiency virus 1 (HIV-1) has a vast evolutionary potential in a human host due to a high rate of mutation (3×10^{-5} substitutions/site/generation) (40), rapid viral turnover (10^8 to 10^9 virions per day) (28, 75), large numbers of infected cells (10^7 to 10^8 infected cells) (10), and a high level of recombination (33). Although the majority of mutations likely have deleterious effects on virus replication and are eliminated by negative selection (20), HIV-1 exhibits remarkable genomic plasticity with very few nucleotide positions conserved across the full-length genome (13). Consequentially, HIV-1 populations may exist as complex mutant swarms termed quasispecies (20, 23). This continual increase in genetic diversity enables HIV-1 to rapidly adapt to a variety of selection pressures. Examples include escape from the humoral (60, 74) and cytotoxic-T-lymphocyte (CTL) (4, 5, 7, 21, 52) immune responses, as well as resistance to all currently available antiretroviral drugs (32).

In the face of strong selection pressure, virus must rapidly adapt to survive. Fitness of an obligate parasite, such as HIV-1, is defined by how these properties affect fecundity (or replication) and survival within a particular host environment (20). These parameters are often difficult to study, but an effective way to examine the replication component of fitness is to compete viral strains in a controlled environment ex vivo (29).

Through the use of these methods, it was demonstrated that viral quasispecies or populations tend to gain fitness with each successive passage in cell culture (12, 48) and that these fitness gains are dependent on increasing population size and genotypic complexity (11). In contrast, any selection pressure that effectively reduces viral population size can also activate Muller's ratchet, in that repeated bottlenecks (e.g., transmission between hosts) lead to fixation of deleterious mutations in the population and a possible loss in fitness (8, 12, 22, 76). Application and relevance of these theories in any viral infection (e.g., HIV-1) of a human host have not been extensively studied.

Several lines of evidence suggest that HIV-1 replication efficiency may correlate with disease progression. Slow progression to symptomatic disease was observed in individuals infected with slow replicating HIV-1 isolates from the same source (39) or with defective virus with *nef* deleted (16, 36). In addition, numerous studies have demonstrated that the transition from a non-syncytium-inducing (NSI) CCR5-utilizing (R5) phenotype to a syncytium-inducing (SI) CXCR4 (X4) phenotype is associated with increased viral replication kinetics (1, 9, 68, 69, 72) and progression to AIDS (14, 61, 62). Even in the absence of a discreet coreceptor change, increased cytopathicity of R5 viruses was found to correlate with a more progressive course of disease (3, 6, 34, 38). We first used this HIV-1 competition-fitness assay in a preliminary study using a limited patient cohort and a cross-sectional sampling approach. These analyses revealed that HIV-1 isolates from long-term survivors had significantly lower ex vivo fitness than HIV-1 isolates from patients who progressed to disease (55).

* Corresponding author. Mailing address: Division of Infectious Diseases, BRB 1029, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106. Phone: (216) 368-8904. Fax: (216) 368-2034. E-mail: eja3@case.edu.

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TABLE 1. Patient clinical information

Patient ^a	Gender	Date (mo/day/yr)		Age (yr) at baseline	Approximate infection date (mo/yr)	ARV treatment ^b drug (start-stop dates)	Complications (yr)	CD4 count	CD8 count	Viral load ^c
		Baseline sample	Last sample					baseline/last sample	baseline/last sample	baseline/last sample
K	Male	7/26/1994	9/20/2000	27	2/1991	None	None	864/412	1,364/1,025	12,201/33,317
R	Male	3/23/1995	12/13/1996	36	3/1993	None	None	318/294	666/1,196	65,631/91,673
I	Male	7/13/1994	5/9/1996	29	5/1991	None	None	422/309	520/294	135,870/134,010
T	Male	10/19/1995	11/29/1999	43	10/1994	None	Nephrolithiasis (1993), onychomycosis (1997)	767/624	1,445/1,742	5,563/49,699
C	Male	3/25/1994	12/4/1995	44	10/1992	None	None	277/269	266/388	154,484/121,503
H	Male	6/17/1994	7/10/1996	33	2/1992	None	None	629/287	2,386/1,474	13,891/31,112
U	Male	9/15/1994	10/22/1998	24	3/1993	AZT (12/96–8/97), 3TC (12/96–10/98), IDV (4/97–8/97), SQV (8/97–9/98), RTV (8/97–9/98), d4T (10/97–10/98), NFV (10/98–10/98)	Anal condyloma, hepatitis (1994), gingivitis (1995), urethritis (1995)	437/620	1,248/1,226	113,622/23,601
M	Male	8/9/1994	8/9/1996	47	1/1987	AZT (10/95–3/97), ddC (12/95–5/96), 3TC (5/96–3/97), ddI (8/96–8/96)	Herpes zoster, old tuberculosis primary infection, hairy leukoplacia (1996), molluscum contagiosum (1996)	355/159	899/696	387,103/579,145
A	Male	3/9/1994	12/20/1996	39	2/1990	AZT (12/90–4/96, 6/96–6/97), ddI (3/96–5/96), ddC (5/96–6/97)	None	248/247	516/594	131,730/45,773
Q	Male	10/31/1994	8/26/1996	33	4/1989	AZT (12/93–12/95, 8/96–9/96), ddC (8/96–3/97)	Cerebral mycobacterial abscess (1994)	262/302	811/1,116	186,553/19,042

^a All patients were ethnically Caucasian.^b Antiretroviral treatment prior to and during the timeperiod of study.^c Viral RNA copies/ml.

In the absence of antiretroviral (ARV) treatment, ex vivo fitness of primary HIV-1 isolates typically maps to the *env* gene and is largely controlled by the efficiency of host cell entry (2, 42, 57).

Mapping of ex vivo HIV-1 fitness to *env* in wild-type virus suggests that evolution in this gene compared to other HIV-1 genes may have a greater influence on virus replication. Although the relationship between HIV-1 *env* genetic diversity and disease progression has been controversial, the most comprehensive study of inpatient HIV-1 *env* evolution found that *env* diversity and divergence from relatively homogeneous founder/infecting virus continued to increase during disease but peaked prior to onset of AIDS (64). Late in disease, genetic diversity and divergence became uncoupled from disease progression (64). The prolonged increase in *env* genetic diversity is ultimately due to mutations but is likely shaped by immune selection, changes in cellular tropism/coreceptor usage, and fitness of the encoded virus (4, 31, 49, 52, 54, 60, 74). To date, there have been few studies comparing relative HIV-1 replication efficiency (ex vivo fitness) to clinical correlates of disease, let alone more comprehensive comparisons to virus diversity. In the present study, we applied rigorous dual HIV-1 competition assays to measure the ex vivo fitness of HIV-1 isolates obtained sequentially from 10 patients over a period of 2 to 5 years. This ex vivo fitness was then compared to various clinical parameters and diversity in the *env* C2V3 region during disease. How HIV-1 replication capacity changes during dis-

ease progression and how this change is related to increases in viral diversity could have fundamental implications as to how this virus ultimately overcomes immune and host selective pressures.

MATERIALS AND METHODS

HIV-1 isolates from the prospective Belgian cohort. Ten patients with typical disease progression (Table 1) were selected from a well-characterized cohort at the Institute of Tropical Medicine in Antwerp, Belgium. At least three different HIV-1 primary isolates from each patient were propagated by coculture of patient peripheral blood mononuclear cells (PBMC) and HIV-negative donor PBMC as previously described (55) and are identified in the present study by the patient letter (i.e., K) and the number of months since the baseline visit, e.g., K11. Virus titer was measured by standard Reed-Muench TCID₅₀ calculations (58). Plasma viral load (RNA copies/milliliter) was determined by using the Amplicor HIV-1 MONITOR test, v.1.5 (Roche).

Sequence analyses. HIV-1 proviral DNA was extracted from the original PBMC of the infected patient by using the QIAamp DNA blood kit (Qiagen). Nested PCR for amplification of the *env* C2V3 region was conducted as previously described (55) with first external primers envB (5'-AGAAAGAGCAGA AGACAGTGGCAATGA-3') and ED14 (5'-TCTTGCCTGGAGCTGTTTGA TGCCCCAGAC-3') and then internal primers E80 (5'-CCAATTCCTATACA TTATTTG-3') and ED33 (5'-TTACAGTAGAAAAATCCCCCTC-3'). PCR products were TA cloned by using pCR2.1-TOPO (Invitrogen) and at least 10 individual clones, representative of 10 *env* C2V3 fragments (nucleotide positions 6903 to 7354 [HXB2 numbering]) from the HIV-1 population, were sequenced for each time point (total of 43 time points for 10 patients). HIV-1 RNA in plasma was extracted by using the QIAampViral RNA Minikit (Qiagen). Reverse transcription reactions were carried out as previously described (55) using primer E105 (5'-GCTTTTCCTACTCTCTGCCAC-3'). At all time points involving ARV therapy and for several without therapy (K, I, and C), the protease-

FIG. 1. Estimation of ex vivo HIV-1 fitness and *env* C2V3 genetic diversity for patient K. An identical strategy was used to estimate ex vivo HIV-1 fitness and genetic diversity for additional patients. (a) Patient PBMC were used to propagate primary HIV-1 isolates for competition and were the source for clonal sequence analysis of the *env* C2V3 region. (b) A representative HTA analysis of competitions between patient K primary isolates (K44 and K69) and NSI/R5 control strains (A-92RW009 and B-92BR017) is shown. K44 and K69 were both able to outcompete the low-fitness control virus A-92RW009, whereas K69 replicated much more efficiently than K44 in competition with B-92BR017. All 34 primary HIV-1 isolates from 10 patients were competed against these NSI/R5 control strains, as well as two SI/X4 control strains. (c) A phylogenetic analysis of *env* C2V3 clonal sequences from each timepoint for patient K was performed by using PAUP* 4.0b10. Colored circles at terminal nodes indicate the sample timepoint from patient K. Significant bootstrap values are indicated on branches (***, >90; **, >80; *, >70). Genetic divergence from the consensus sequence at the first timepoint for each patient is shown as synonymous (d_S) and nonsynonymous (d_N) mutations. The d_S/d_N ratio within each set of clonal sequences at each time point is also displayed.

reverse transcriptase (RT) region of the *pol* gene (nucleotides 2280 to 3206 [HXB2 numbering]) was PCR amplified from patient PBMC DNA as previously described (59) and then sequenced. Nucleotide sequencing of the *env* and *pol* regions was performed by Davis Sequencing, Inc., using an ABI 3700 DNA sequencer. The *env* C2V3 and *pol* nucleotide sequences reported in the present study have been submitted to GenBank under accession numbers DQ61397 to DQ61399 and DQ61397 to DQ61399, respectively.

Nucleotide sequence alignments for both the average *pol* sequences and the sequences for all of the *env* clones were produced with CLUSTALX v.1.83 (70) and then manually edited for codon alignment. Pairwise genetic distance was determined with the MEGA 2.1 program (37) by using the Kimura two-parameter model (35), while synonymous (d_S) and nonsynonymous (d_N) mutations were calculated by using the method of Nei and Gojobori (46). Divergence was calculated as the pairwise genetic distance of all clones at a given time point to the consensus sequence at the first time point for each patient. An interpatient neighbor-joining phylogenetic analysis of all 454 *env* C2V3 clones was performed with 1,000 bootstrap resamplings by using PHYLIP v.3.5 (24) to confirm a lack of cross-sample contamination. Phylogenetic trees of the HIV *env* clonal sequences from each patient were constructed with the PAUP* 4.0b10 (67) program by using the alignments generated in CLUSTAL X. These trees were constructed by initially estimating neighbor-joining relationships using maximum-likelihood distances and then swapping branches by using the tree bisection-reconnection algorithm for all patients. Due to the number of *env* clones and sequence length, phylogenetic trees for patient K were constructed by using the subtree pruning-grafting algorithm. Models of sequence evolution were estimated directly from the data under a general time-reversible model allowing for

among-site rate variation, various nucleotide frequencies, and transition/transversion ratios. The significance of the branching order was estimated by bootstrap resampling of 1,000 replicates.

Ex vivo competition assays and determination of coreceptor usage. The methods used for determining ex vivo HIV-1 fitness have been previously described in detail (55). Briefly, each HIV-1 primary isolate was added to phytohemagglutinin (2 μ g/ml)- and interleukin-2 (1 ng/ml)-treated PBMC, along with each of four primary HIV-1 control strains: two NSI/R5 HIV-1 isolates (A-92RW009 and B-92BR017) and two SI/X4 isolates (A-92UG029 and E-CMU06), all at a multiplicity of infection (MOI) of 0.0001. An RT activity assay was performed on supernatant from each dual infection (71) to determine virus production. Cells and supernatant were harvested and stored at -80°C on day 12.

Coreceptor usage (CCR5 and CXCR4) of each HIV-1 isolate was determined by duplicate infection of 100,000 U87.CD4-CCR5 and U87.CD4-CXCR4 cells at an MOI of 0.0004 as previously described (71). Coreceptor usage was then compared to that predicted from the V3 *env* genotype by using a bioinformatic method that predicts X4 usage by scoring all V3 amino acid positions using position-specific scoring matrices (PSSM) (31).

HTA and estimation of viral fitness. A sensitive and quantitative heteroduplex tracking assay (HTA) was used to differentially quantify virus production of individual HIV-1 isolates in dual-infections as previously described (55). Briefly, the C2V3 region of *env* was amplified from the PBMC DNA of all mono-infections and dual infections by using conserved primer pairs (Fig. 1). The *env* region from subtype A, E, and D clones was amplified with a 5'-³²P-end-radiolabeled primer for use as DNA probes. At least two DNA probes in separate HTAs were used to determine relative virus production in each dual infection. Probes and

TABLE 2. Coreceptor usage of HIV-1 primary isolates

Primary HIV-1 isolate	Presence (+) or absence (–) of infection in:		Amino acid ^a at position:		Coreceptor usage
	U87-R5	U87-X4	306	322	
K11	+	–	S	E	R5
K44	+	–	S	E	R5
K69	+	+	S/R	E	R5/X4
K74	+	+	S/R	E	R5/X4
T0	+	–	G	E	R5
T21	+	–	G	E	R5
T46	+	–	G	E/A	R5
T49	+	–	G	E/A	R5
R0	+	–	S	D	R5
R13	+	–	S	D	R5
R21	+	–	S	D	R5
I0	+	–	S	E/D	R5
I10	+	–	S	E/D	R5
I22	+	–	S/G	E/D	R5
C0	+	–	S	E	R5
C11	+	–	G/S	E	R5
C20	+	+	G/S	E/K/R	R5/X4
H0	ND	ND	S	E	R5
H10	+	–	S	E/G	R5
H25	+	–	S	E	R5
U0	+	–	G	Q	R5
U11	+	–	G	Q	R5
U24	+	–	G	Q	R5
U49	ND	ND	G	Q	R5
M0	+	+	L/S/R	R/del	R5/X4
M12	+	+	S/H	R/K/del	R5/X4
M24	+	+	S/H	R/del	R5/X4
A0	+	–	S	E	R5
A11	+	–	S	E	R5
A25	+	–	S	E	R5
A33	+	–	S	E	R5
Q0	+	–	G	G/A	R5
Q13	+	–	G	A	R5
Q22	+	–	G	A/N/D	R5

^a Amino acid residues at position 306 or 322 of the *env* gene are given. Positions are based on the HXB2 sequence. Basic residues (in boldface) at positions 306 and 322 of *env* are strongly associated with CXCR4 coreceptor usage. ND, not determined due to insufficient quantities of primary HIV-1 isolates.

PCR products were mixed, denatured, annealed, and then separated on a non-denaturing 6% polyacrylamide gel. Heteroduplexes corresponding to each virus in a given competition were quantified by using a Molecular Imager FX (Bio-Rad) phosphorimager (Fig. 1). The final ratio of the two viruses produced from each dual infection, the relative fitness (w), was determined by comparing virus production in the competition to virus production in the monoinfections. Production of individual HIV-1 isolates in a dual infection (f_0) was divided by the initial proportion in the inoculum (i_0) and is referred to as relative fitness ($w = f_0/i_0$). The ratio of the relative fitness values of each HIV-1 isolate in the competition is a measure of the fitness difference (W_D) between the two HIV-1 variants ($W_D = w_M/w_L$), where w_M and w_L correspond to the relative fitness of the more and less fit virus, respectively.

RESULTS

Relating ex vivo HIV-1 fitness to clinical parameters of disease progression. We examined the changes in ex vivo HIV-1 fitness and HIV-1 genetic diversity during disease progression in 10 patients (indicated with the capital letters K, R, I, T, C, H, U, M, A, and Q) from a well-characterized Belgian cohort. All 10 patients were essentially normal progressors at various stages of disease progression (Table 1). Approximate

seroconversion dates were based on routine HIV-1 screening in an uninfected patient cohort attending this clinic and then enrolling the patients into the present study sometime after seroconversion. Primary HIV-1 isolates were propagated from at least three blood samples collected over a period of 2 to 5 years and were identified by the patient letter (i.e., K) and the number of months since the baseline visit, e.g., K11 (Fig. 1). It is important to note that propagation of primary HIV-1 isolates from PBMC cocultivations does result in a narrowing of the quasispecies diversity and some clonal selection. Previous findings, however, suggest that the HIV-1 clones selected during in vitro propagation are often the same as those subsequently selected within the host at a later time (18, 66). Similar phylogenetic structures were observed in the *env* gene of the propagated viruses and the HIV-1 sequences of plasma and PBMC samples at the same time point (see Fig. S5 in the supplemental material).

Each virus isolate was added to U87.CD4.CCR5 (or CXCR4) cells to determine coreceptor usage, as well as monitor replication kinetics in a monoinfection. Coreceptor usage was identical to that predicted in the propagated virus based on the charge of the average *env* V3 sequence, the presence of a basic amino acid at position 306 or 322 of *env* (Table 2), and the use of PSSM (31). As outlined below, only 6 of the 34 propagated HIV-1 isolates were characterized as dualtropic (X4/R5), and none displayed a pure X4 phenotype. Of the six dualtropic viruses, only two X4 isolates (K69 and K74) emerged from an obvious mixture of R5 and X4 clones in the virus population found in the patient PBMC that was then used for propagating the patient virus sample. For the remaining four X4/R5 dualtropic viruses or the R5 HIV-1 isolates, the virus clones found in the original PBMC were all predicted to be CXCR4- or CCR5-tropic viruses, respectively. Based on previous studies, it is likely that we did not sufficiently sample the virus population in PBMC to find X4 (or R5) clones among the dominant R5 (or X4) virus population. Regardless, these findings do suggest that during propagation of virus from an asymptomatic time point, a minor population of X4 clones may not as yet have attained the replicative fitness to rapidly emerge and dominate over the R5 clones (50).

Ex vivo fitness values were then estimated for all primary isolates by performing dual-infection competitions with four HIV-1 competitor strains in HIV-negative PBMC. Because ex vivo fitness values were expected to vary widely (2, 55), competitor strains were chosen to represent a range of fitness levels. These competitors included two NSI/R5 viruses (A-92RW009 and B-92BR017) and two SI/X4 viruses (A-92UG029 and E-CMU06) (2, 55). As indicated previously (55), these reference strains were selected as reference strains from 12 primary HIV-1 isolates used in a previous pairwise competition analyses. The reference strains span the gamut of fitness differences, i.e., a fitness order of A-92RW009 < B-92BR017 < E-CMU06 < A-92UG029. A previous study found that an equal ratio of primary isolate to competitor virus was sufficient to determine fitness differences, so all competitions were performed at 1:1 ratios of infectious virus (MOI of 0.0001) (55). Production and fitness of each virus in a dual infection was monitored by using a sensitive HTA and compared to that in a PBMC monoinfection (Fig. 1). Quantitation of dual virus production by HTA has been validated by using both a cloning/dot

FIG. 2.

FIG. 3.

FIG. 4. Interpatient correlates of ex vivo HIV-1 fitness. Pearson product moment correlations were determined for all 34 HIV-1 primary isolates analyzed in the present study. Ex vivo HIV-1 fitness correlated with time since first positive HIV test (a), genetic diversity (b), CD4 cell count (c), and plasma viral load (d). The 95% confidence intervals for the line are displayed.

blot approach (55) and TaqMan real-time PCR (73). It is important to note that all mono- and dual infections were performed with PBMC of the same donor and blood draw. A subset of these competitions was performed on PBMC from

two other donors with results nearly identical to those described below (data not shown). Figure 1 provides a sample of competition/HTAs and indicates that the month 69 virus of patient K (K69) had a greater fitness than the month 44 virus

FIG. 2. Trends in HIV-1 ex vivo fitness, genetic diversity, and disease progression for six ARV naive patients (panels a to f). The time scale is shown in months with the baseline (zero) time point indicating the first available sample for each patient. The number in parentheses at the baseline time point indicates the number of months from the patient's first positive HIV test to the first sample. Vertical bars indicate the total relative fitness for each HIV-1 primary isolate derived from competitions against the four control strains. Each bar is marked with the HIV-1 isolate name (by patient and month, e.g., K11 and K44) and coreceptor usage is shown by the color of the bar: light gray, R5; dark gray, R5/X4. The CD4 cell count is indicated with red closed circles, whereas the plasma viral load is indicated with green open circles. Genetic diversity and divergence are indicated in blue with squares and triangles, respectively.

FIG. 3. Trends in HIV-1 ex vivo fitness, genetic diversity, and disease progression for four ARV experienced patients (panels a to d). The time scale is shown in months, with the baseline (zero) time point indicating the first available sample for each patient. The number in parentheses at the baseline time point indicates the number of months from the patient's first positive HIV test to the first sample. Vertical bars indicate the total relative fitness for each HIV-1 primary isolate derived from competitions against the four control strains. Each bar is marked with the HIV-1 isolate name (by patient and month, e.g., U0, U11, etc.), and coreceptor usage is indicated by the color of the bar: white, R5; gray, R5/X4. The CD4 cell count is indicated with red closed circles, while the plasma viral load is indicated with green open circles. Genetic diversity and divergence are indicated in blue with squares and triangles, respectively. Patients received antiretroviral treatment for the time periods indicated by bars next to the drug name: AZT, zidovudine; 3TC, lamivudine; ddI, didanosine; ddC, zalcitabine; d4T, stavudine; IDV, indinavir sulfate; SQV, saquinavir mesylate; RTV, ritonavir; NFV, nelfinavir. Patients A and Q received AZT treatment for 39 and 10 months prior to the zero time point, respectively.

(K44) since the K69 could more efficiently compete with the control competitor viruses A-92RW009 and B-92BR017. The total relative fitness of each patient isolate (34 in total) was obtained from competition against each of four control viruses. Previous studies suggest that recombination does not affect fitness determination since the frequency of HIV-1 recombination was ca. 1% of the dual virus production or below the limit of HTA detection (55, 56).

Monoinfections limited to a single round of replication appear to be somewhat correlative to relative fitness derived from competitions (53). However, we and others have found (2, 55) that in the absence of a severely debilitating defect (e.g., *nef* deletion) (15), virus replication kinetics derived from monoinfections (involving multiple cycles of replication) are poor predictors of competitive replicative fitness. In the present study, replication kinetics in monoinfections did vary among the 28 R5 and 6 dual R5/X4 HIV-1 isolates but again did not correlate with ex vivo fitness values (Fig. S1 in the supplemental material). It is important to note that all viruses with the exception of virus from patient A did not contain drug resistance mutations. Head-on competitions in cell culture between two viral isolates provides the internal control lacking in monoinfections and is more sensitive in discerning small differences in replication efficiencies. The inherent variation between culture conditions, even of the same cells infected on the same day with different viruses, as well as the nature of the virus detection assays (41), is sufficient to obscure small fitness differences. Finally, the relative fitness values derived from specific competitions are highly reproducible and represent a relative measure (2, 54, 55, 57, 73) rather than an absolute production over time as with monoinfections.

Figures 2 and 3 summarize clinical data (viral load and CD4 cell count) monitored over a 2- to 6-year time interval for the 10 patients, along with measurements of ex vivo fitness. Patients K, R, I, T, C, and H had not received ARV treatment (Fig. 2). Patients U and M received ARV treatment 27 and 14 months, respectively, from the collection of the first sample (Fig. 3a and b), whereas patients A and Q received therapy prior to and throughout the study interval (Fig. 3c and d). Fitness of HIV-1 isolates during disease progression in these asymptomatic patients followed a noticeable trend, i.e., total relative fitness increased during time of infection. The hypothesis that HIV disease progression may be, at least in part, related to viral replicative fitness requires that ex vivo HIV-1 fitness correlate with known markers of disease progression. This was indeed the case, as ex vivo fitness significantly correlated with both CD4 cell count (Fig. 4c, $r = -0.443$, $P = 0.009$, Pearson product moment correlation) and plasma viral load (Fig. 4d, $r = 0.486$, $P = 0.004$, Pearson product moment correlation). On an individual patient basis (9 of 10 patients), a direct relationship was observed between ex vivo HIV-1 fitness and plasma viral load that was significant based on analyses of mean slope (Fig. 5, $P = 0.023$, one-sample t test, outlier excluded). Eight of ten patients exhibited an inverse relationship between ex vivo HIV-1 fitness and CD4 cell count, although the mean trend was not statistically significant (Fig. 5, $P > 0.05$). This relationship of fitness with viral load (direct correlation), CD4 cell count (inverse trend) and with genetic diversity (direct correlation), as described below, appear to be independent of ARV treatment. Fitness of the patient M viruses

FIG. 5. Intrapatient correlates of ex vivo HIV-1 fitness. Slopes of the correlation between ex vivo HIV-1 fitness and genetic diversity, plasma viral load and CD4 cell count were plotted for each patient ($n = 10$). The significance of the mean slope of each correlation (null hypothesis = 0) was tested by using a one-sample t test. Outlier points are displayed as open circles, and analyses excluding these points are displayed.

were the highest among all patient samples attesting to their time of isolation, i.e., late in disease progression. These fitness values are having significant contribution to the correlation with CD4 cell count, viral load, and genetic diversity. Nonetheless, when the patient M datum points were removed all correlations with fitness remained significant (fitness versus CD4 cell count, $r = 0.36$, $P < 0.04$; fitness versus diversity, $r = 0.50$, $P < 0.001$) except fitness and viral load ($r = 0.28$, $P < 0.2$).

We initially used competitions against reference strains rather than competitions between viruses of the same patient. The general difficulty of competing primary HIV-1 isolates of the same patient is first determining if the sequence difference is sufficient to detect two heteroduplexes (often two mixtures of distinct heteroduplex bands) on a polyacrylamide gel. Viruses from patients T and I were some of the few with sufficient *env* diversity between sequential virus samples to permit direct competitions and accurate quantitation of relative fitness values based on HTA (Fig. 6a). In addition, we had limited quantities of the initially propagated viruses to perform competitions. As outlined previously, viruses T0, T21, I0, I10, and I22 were all competed against the control strains (Fig. 6c and e). Viruses T0 and T21 had low fitness, T21 being more fit than T0, as indicated by their ability to only compete against NSI/R5 A-92RW009. All other control viruses completely outcompeted these two isolates. When competed together in head-to-head competition, T21 dominated the competition over T0 (Fig. 6d). T21 being more fit than T0 in direct competition reflects the difference in fitness derived from competitions with A-92RW009. Interestingly, the heteroduplexes that identified each virus population at T0 and T21 migrated to distinct positions on the gel. The apparent homogeneity of each virus-specific heteroduplex band reflects the limited diversity observed in the PBMC samples at months 0 and 21 but a

FIG. 6. Comparing fitness of primary isolates from the same patient in head-to-head competitions. HIV-1 isolates from patient T at 0 and 21 months (i.e., ca. 12 and 33 months after infection) were competed together in a PBMC culture as described in Materials and Methods. HTA was performed by using the C4 HIV-1 probe. Heteroduplex bands representing virus T0 and T21 in mono-infections and direct dual infections are shown in panel (a). (b) A phylogenetic analysis of *env* C2V3 clonal sequences from each time point for patient T was performed by using PAUP* 4.0b10. Shaded circles at terminal nodes indicate the sample time point from patient T. Significant bootstrap values are indicated on branches (***, >90; **, >80; *, >70). The boxes highlight terminal nodes from the HIV-1 T0 and T21 sequences within the patient PBMC, i.e., the same PBMC sample used to propagate the viruses. The results of T0 and T21 (c) or I0, I10, and I22 (e) competitions against control strains are presented as individual pie charts. The gray slices represent the percentage of sample virus growth against one of the four control strains, which are represented by black slices (A-92RW009, B-92BR017, A-92UG029, and E-CMU06). Total relative fitness values (W) derived from control virus competitions are indicated in panels c and e. The results from direct competitions between T0 and T21 are shown in panel d. Competitions I0 versus I22 and I10 versus I22 are shown in panel f. Gray and black slices of each pie chart in panels d and f are as labeled in the figure.

significant divergence between these patient T samples (Fig. 6b).

Both T0 and T21 had fitness values in the lower range of all of the patient isolates analyzed, but the difference in fitness between T0 and T21 was great enough to result in the complete outgrowth of T21 over T0 in direct competition (Fig. 6d). With patient I, virus samples from months 0, 10, and 22 had modest differences in viral fitness that appeared to correspond to stable CD4 count and viral load (Fig. 2c). The differences in diversity and migration of the heteroduplex bands were insufficient between viruses I0 and I10 to permit direct competition. However, I22 had diverged from these viruses to permit direct I0 versus I22 and I10 versus I22 competitions and detection by HTA. Unlike patient T viruses, viruses I0, I10, and I22 dominated competitions against A-92RW009 and could compete with B-92BR017 (Fig. 6e). Although the viral fitness increased

over time, the fitness differences between I0, I10, and I22 were not great when competed against control strains. As a result, head-to-head competitions showed that I22 was more fit than both I0 and I10 but did not completely dominate (Fig. 6f).

Comparing ex vivo HIV-1 fitness, viral diversity, and parameters of disease progression. In order to characterize the genetic diversity and divergence of HIV-1 in each patient, clonal sequencing of the *env* C2V3 region was performed (Fig. 1). The *env* C2V3 region was chosen for this analysis because the efficiency of virus entry appears to play the predominant role in controlling ex vivo HIV-1 fitness (2, 57). The central role of the V3 loop in virus entry further suggests that this region may have a significant influence on ex vivo HIV-1 fitness. The HIV-1 genetic diversity at each time point was estimated as the mean pairwise genetic distance among at least 10 *env* C2V3 clones, whereas genetic divergence was estimated as

the pairwise genetic distance of all clones at a given time point to the consensus sequence at the first time point for each patient. A phylogenetic analysis of all 454 *env* C2V3 clonal sequences demonstrated that all sequences clustered together by patient with significant bootstrap support, confirming the lack of interpatient cross-contamination of samples (Fig. S2 in the supplemental material). Intrapatient phylogenetic trees demonstrated variable patterns of divergent evolution (Fig. 1; see also Fig. S3 and S4 in the supplemental material). Phylogenetic analyses of these *env* C2V3 clones and average *pol* sequences demonstrated that each patient was infected with subtype B HIV-1 (data not shown). In addition, a phylogenetic analysis of average *env* C2V3 sequences derived from patient PBMC, plasma, and propagated virus was performed in order to compare the dominant virus populations present in each of these sources. This analysis demonstrated a highly similar composition of the average sequence (Fig. S5 in the supplemental material). Diversity of the HIV-1 *env* C2V3 region increased during asymptomatic disease and continued to diverge from the founder virus, as was the case in the Shankarappa et al. (64) study. The relatively high viral loads in all patient samples ($>10^3$ copies/ml) ensured ample viral RNA or DNA (in PBMC) for PCR amplification and reduced the possibility of sampling error. Two independent PCR amplifications, cloning, and sequencing of 10 clones from the patient sample I0 (lowest viral load) were performed to test for possible sampling error. Nearly identical sequence diversity among the clones in this population and similar mutational patterns suggests appropriate sampling of the HIV-1 population (data not shown).

The *env* C2V3 genetic diversity and divergence was then compared to ex vivo fitness of sequential primary HIV-1 isolates, as well as the clinical correlates of disease progression. Although direct and inverse correlations with viral load and CD4 cell counts were not significant ($P > 0.05$, Pearson; data not shown), the correlation between viral quasispecies diversity and ex vivo fitness was highly significant ($r = 0.652$, $P < 0.0001$, Pearson; Fig. 4b). This relationship remained significant if only nonsynonymous mutations (d_N) or synonymous mutations (d_S) were considered ($r = 0.650$, $P < 0.0001$; $r = 0.595$, $P = 0.0002$, respectively; data not shown). In the absence of datum points derived from patients treated with ARVs, the correlations between relative fitness with viral loads or diversity remained significant ($r = 0.490$, $P = 0.01$ and $r = 0.600$, $P = 0.0015$, respectively). Only the relationship between viral fitness and CD4 cell counts fell slightly below significance ($r = -0.364$, $P = 0.07$). In order to quantitatively assess this relationship on an intrapatient basis, we compared the slopes derived from plots of ex vivo HIV-1 fitness and HIV-1 genetic diversity for each patient and found that in 9 of 10 patients, there was a positive relationship ($P = 0.086$, Fig. 5). The exception, patient M, displayed a slight inverse relationship between ex vivo fitness and genetic diversity but was also in the latest stages of disease (VL $> 600,000$ copies/ml, CD4 cell count < 190 , dual X4/R5-tropic virus). When patient M was excluded as an outlier, the mean intrapatient correlation between ex vivo fitness and genetic diversity was statistically significant ($P = 0.004$, Fig. 5).

In the absence of ARV therapy, the ex vivo fitness of sequential HIV-1 isolates consistently increased during the time of infection (16 of 17 sequential isolations, Fig. 2 and 3). Thus, it was of interest to determine whether there might be a cor-

relation between ex vivo HIV-1 fitness and length of infection in an intrapatient analysis. Exact dates of seroconversion were not available for the majority of patients, so the date of first positive HIV test with previous negative tests as a reference was used to determine an estimated date of seroconversion. There was a striking correlation between ex vivo HIV-1 fitness and time since first positive HIV-1 test (Fig. 4a, $r = 0.683$, $P < 0.0001$, Pearson), suggesting that replication efficiency may be related to the length of infection. Finally, plots of ex vivo fitness over time in each patient can almost be superimposed on plots of *env* diversity over time, suggesting a dependent relationship between fitness and diversity ($r = 0.881$, $P < 0.001$, Spearman rank correlation of fitness and diversity slopes over time). The month 25 sample of patient H (Fig. 2f) was the only case in which ex vivo fitness decreased in the absence of ARV therapy. Notably, this decrease in ex vivo fitness was associated with a sharp decrease in *env* genetic diversity. A phylogenetic analysis of the *env* C2V3 population at this month 25-time point showed the emergence of a divergent but nearly homogeneous population of virus (Fig. S3a in the supplemental material). As described above with other clinical parameters, fitness of patient M viruses had a positive impact on the correlation with infection time length. However, this correlation remained significant even when these patient M datum points were removed ($r = 0.55$, $P < 0.001$). In the discussion, we surmise that the concomitant increases in fitness and HIV diversity during disease may be interrelated and based on selection of more fit, host-adapted variants. However, this diversity may result in immune escape HIV-1 variants that are selected due to HIV-specific host immune pressure. Preliminary studies suggest that immune escape comes with a viral fitness cost, but few studies have directly tested this hypothesis.

Possible fitness and genetic bottleneck imposed by ARV treatment. Introduction of ARV therapy in patients M and U during the course of disease/sample collection coincided with a decrease in ex vivo HIV-1 fitness (patient U, Fig. 3a; patient M, Fig. 3b). For patient U, the decrease in ex vivo fitness and stabilization of genetic diversity and divergence was observed after 2 years of ARV therapy. Multiple regimens of suboptimal therapy resulted in limited control of viremia and a modest increase in CD4 cell counts. Patient M had characteristics of late-stage disease/AIDS, including a high viral load ($>600,000$ copies/ml), a CD4 count below 200, CXCR4 coreceptor usage, and the highest ex vivo HIV-1 fitness in the present study. ARV therapy with nucleoside reverse transcriptase inhibitors was not effective in altering CD4 count or plasma viral load, and yet the initiation of therapy coincided with a moderate decrease in ex vivo HIV-1 fitness. Patients A and Q, who received ARV therapy during the course of study, exhibited fluctuating ex vivo HIV-1 fitness (Fig. 3c and d). With the exception of one time point, these fluctuations appeared to correspond to respective changes in HIV-1 genetic diversity in both patients.

The potential impact of ARV resistance on ex vivo fitness was examined by PCR amplifying *pol* from patient PBMC DNA and sequencing a region containing the majority of primary nucleoside reverse transcriptase inhibitor and protease inhibitor resistance mutations (protease amino acid 10 through RT amino acid 220). *pol* sequences from all ARV-associated time points of patients U, M, and Q indicate the lack of any

ARV resistance mutations (Table S1 in the supplemental material). In addition, there were no drug resistance mutations found in the *pol* gene of the untreated patient samples (Table S1 in the supplemental material). Patient A, who was treated with zidovudine (AZT) for 39 months prior to baseline (time = 0) in the present study, harbored HIV-1 containing the AZT resistance conferring mutations M41L and T215Y in RT at all time points (Table S1 in the supplemental material). These mutations may confer a negative impact on fitness (19, 26). Interestingly, the relationship between ex vivo fitness, viral load, and viral diversity (Fig. 3c) did not appear to be affected by the presence of these AZT-resistant mutations, suggesting compensatory mutations during three years of AZT monotherapy. The lack of other HIV-1 drug-resistant mutations in the patient U, M, and Q PBMC samples was a bit surprising since all were on suboptimal treatment regimens. However, it is possible that drug-resistant mutation had emerged and simply faded into the HIV-1 population due to frequent changes in ARV treatments. Alternatively, virus population placed through a weak genetic bottleneck due to weak antiviral activity of drug does not necessarily require the emergence of drug resistant mutations for continued virus replication (45).

Relationship between coreceptor usage and ex vivo fitness. Coreceptor usage was measured on U87 brain glioma cells expressing CD4 and either CCR5 or CXCR4. Overall, R5/X4 dualtropic viruses were more fit in PBMC cultures than viruses that exclusively utilized CCR5 as coreceptor ($P < 0.001$, Student *t* test) and a switch from R5 to R5/X4 viruses in patients K and C resulted in a notable increase in fitness. Although this increase in ex vivo fitness was associated with a switch in coreceptor usage (R5 to R5/X4), the correlation of ex vivo HIV-1 fitness with genetic diversity ($r = 0.560$, $P = 0.002$), time since first positive HIV test ($r = 0.670$, $P < 0.0001$), and CD4 count ($r = -0.390$, $P = 0.04$) remained significant when data related to the time points harboring the R5/X4 virus were removed. Only the relationship between plasma viral load and ex vivo fitness was no longer significant ($r = 0.295$, $P = 0.13$).

We analyzed HIV-1 V3 sequence derived from patient PBMC, plasma, or the propagated HIV-1 isolates (used for ex vivo fitness determination) by using a PSSM approach (31). This score has been shown to vary continuously with the ability of the isolate to use CXCR4 for cell entry. The transition between R5 and X4 is likely a gradual multistep process (25, 31, 50, 72), and signature sequences in this transition may be related to increases in ex vivo fitness even in the absence of a discrete change in tropism. Considering all time points for which sequence and fitness data were available ($n = 34$), we found strong correlations between fitness and average PSSM scores in HIV-1 harbored in patient PBMC ($r = 0.589$, $P < 0.0002$; Fig. 7a) and scores from the propagated isolate ($r = 0.780$, $P < 0.0001$; Fig. 7b). An analysis of variance comparing fitness as response variable to patient and monoculture PSSM scores as factors indicated that both exerted strong and independent influence on fitness ($F_{\text{patient}} = 144.42$, $P < 0.002$; score $F_{\text{score}} = 6.30$, $P < 0.0001$; interaction $F = 2.04$, $P = 0.11$). However, we found no correlation between fitness and PSSM scores of plasma virus sampled at the same time points ($P = 0.91$). This was in spite of strong correlations among PBMC, monoculture, and plasma virus scores themselves and the obvious sequence relationships found in a neighbor-joining

FIG. 7. Competitive fitness versus PSSM score. The total relative fitness of each primary HIV-1 isolate was compared to the PSSM score based on V3 sequences from patient PBMC (a) and the propagated primary HIV-1 isolate (b). The raw PSSM scores were normalized by mapping the minimum score to -1 , the maximum score to 1 , and intermediate scores to points between -1 and 1 reflecting their original fractional position between the minimum and maximum.

phylogenetic analysis (Fig. S5 in the supplemental material). These data suggest that a switch in coreceptor usage is responsible for significant increases in fitness. However, it is important to note that the fitness of NSI/R5 HIV-1 isolates also continued to increase during disease progression and prior to this switch.

DISCUSSION

Ex vivo HIV-1 fitness is likely to be dependent on the cellular microenvironment (fitness landscape) (20), but how this fitness applies to survival and expansion in a patient has not been extensively studied. In the present study competition between the primary HIV-1 isolates and four control strains

provided a reproducible measure of relative replication efficiency (e.g., ex vivo fitness) in PBMC, which was not discernible in monoinfections. Although it is certainly impossible to model the full complexity of the human ecosystem in any exogenous replication system, ex vivo HIV-1 fitness in human PBMC provides a surrogate measure of replication efficiency. This ex vivo HIV-1 fitness in human PBMC was found to correlate strongly with clinical parameters (plasma viral load and CD4 cell count) of disease progression, as well as the approximate length of infection (3 to 9 years) in 10 patients. The strong relationship between this fitness and HIV-1 *env* C2V3 genetic diversity further suggests that genotypic changes and probing of HIV-1 sequence space could lead to phenotypic variations. In fact, each minor fluctuation in ex vivo fitness appeared to be reflected by a direct and relative change in diversity. Although these findings were based on a limited number of patients ($n = 10$) with samples collected over a limited period of time (2–5 years), the present study is the most extensive to date characterizing both ex vivo HIV-1 fitness/replication capacity and genetic diversity during disease progression. It should also be noted that even minimal propagation could result in a narrowing of the quasispecies diversity and some ex vivo selection of specific clones. However, our observations based on sequencing of clonal populations in the blood (e.g., PBMC, plasma, and propagated viruses) are consistent with reports that the limited clonal selection is evident due to increased fitness. These clones often dominate at the time of sample collection or rapidly emerge in the patient virus population at later time points (18, 66). Nonetheless, our phylogenetic analyses of the HIV-1 *env* gene and of PSSM scores suggest a highly similar genetic composition of the HIV-1 quasispecies in patient PBMC, plasma, and propagated virus.

The relationship between ex vivo fitness and approximate length of infection supports previously proposed models (1, 3, 9, 34, 38, 51, 54, 55) in which HIV-1 evolves greater pathogenicity due to increasing replicative fitness. This increasing pathogenicity may be directly related to relative HIV-1 replication in cells (2, 3, 27, 34, 38, 55, 63) or indirectly related through increasing immune dysfunction (17, 27, 44). Many studies suggest that the switch from slower-replicating R5 to the dualtropic or purely X4 virus with higher replicative capacity is largely responsible for this increased pathogenicity (1, 3, 9, 34, 62). Although we have observed that fitness increases over time even in patients infected with R5 viruses, a switch in tropism did significantly increase ex vivo fitness. A PSSM sequence analysis (31) of the V3 region revealed that the plasma- and PBMC-associated viruses directly from the patient had similar X4 versus R5 signature sequences, as did the propagated virus, and that PSSM scores of PBMC and propagated virus correlated with fitness. The reason why plasma scores were not correlated with fitness is likely related to the underrepresentation of dualtropic viruses in plasma. The correlation between fitness and PSSM score, which is weighted on specific V3 amino acid sequences, strongly suggests that specific changes in V3 play a role in controlling viral fitness. Put another way, the correlation implies that 60% of fitness variance among the isolates is related to changes in V3. Finally, the deterministic role *env* plays in controlling ex vivo fitness suggests a plausible cause-effect relationship between *env* genotypic changes and phenotype (e.g., relative replication effi-

ciency; Fig. 2 and 3). Nonetheless, we cannot rule out positive fitness effects of changes elsewhere in the genome.

In the present study, it appears that ARV treatment may create a bottleneck to limit expansion of the HIV-1 population. These data confirm previous studies which have found that *env* diversity is restricted by suppressive ARV therapy (30, 43). Eventual escape from this bottleneck and the emergence of drug-resistant viruses comes at a cost to the virus. Several reports suggest that drug-resistant viruses have reduced replication kinetics compared to wild-type strains (26, 47, 65). During disease progression and in the absence of drug pressure, selective pressure is likely due to both innate and acquired immune responses. Both humoral and cell-mediated HIV-specific immune responses select for escape mutations in specific viral epitopes (4, 5, 7, 21, 52, 60, 74), i.e., in principle, escape similar to that observed with drug-resistant mutations. In the present study, samples were only available at least 1 year after acute infection and thus most escape from CTL responses may have emerged prior to the time of study. However, the infecting virus would still be subject to humoral and some CTL selective pressure during this 2- to 6-year span of infection. In the case of strong drug selective pressure and subsequent escape (i.e., drug resistance), there is a substantial decrease in replicative capacity (26, 32, 47). In contrast, the humoral and/or CTL response after early infection does not appear to have a negative impact on virus replication capacity since fitness (as measured by our ex vivo assays) continues to increase during disease progression. Although escape mutations have been readily identified in both humoral and CTL epitopes throughout disease (4, 5, 7, 21, 52, 60, 74), it would appear that these mutations may not have as significant of an impact on virus replication kinetics (ex vivo fitness) or constricting genetic diversity as do drug-resistant mutations. Continual virus replication through a “wider” bottleneck will permit more evolution and possible compensation of detrimental mutations (e.g., humoral escape mutations) (8, 11, 20, 48, 76). Only in long-term nonprogressors has there been evidence of substantial control of viremia by HIV-specific immune responses. Interestingly, the virus harbored by these patients was found to be significantly less fit than HIV-1 isolates from typical progressors (55). Ability to detect dips in HIV-1 fitness during disease may simply require more frequent sampling but, nonetheless, any decreases in fitness appear to be less dramatic than that observed during drug treatment and may be rapidly compensated for by secondary mutations.

In conclusion, we found that ex vivo HIV-1 fitness correlated strongly with HIV-1 *env* C2V3 genetic diversity, suggesting that these parameters may be linked. As HIV-1 diverges and diversifies, it explores the fitness landscape, allowing for progressive adaptation to a state of greater replicative fitness, even in the absence of a coreceptor switch. ARV treatment appeared to alter this pattern, even in the absence of primary resistance mutations, suggesting that ARV therapy may shift the dominant selection pressure to the drug targets, e.g., RT and protease. The strong correlation of ex vivo HIV-1 fitness with disease progression supports the hypothesis that the fitness or pathogenic potential of HIV-1 isolates establishing acute infection could predict the subsequent rate of immune destruction (CD4 depletion), the strength of HIV-1 specific response (CTL or T-helper-cell response), and disease progression.

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