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A Dual Infection/Competition Assay Shows a Correlation between Ex Vivo Human Immunodeficiency Virus Type 1 Fitness and Disease Progression

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This study was designed to examine the impact of human immunodeficiency virus type 1 (HIV-1) fitness on disease progression through the use of a dual competition/heteroduplex tracking assay (HTA). Despite numerous studies on the impact of HIV-1 diversity and HIV-specific immune response on disease progression, we still do not have a firm understanding of the long-term pathogenesis of this virus. Strong and early CD8-positive cytotoxic T-cell and CD4-positive T-helper cell responses directed toward HIV-infected cells appear to curb HIV pathogenesis. However, the rate at which the virus infects the $CD4^+$ T-cell population and possibly destroys the HIV-specific immune response may also alter the rate of disease progression. For HIV-1 fitness studies, we established conditions for dual HIV-1 infections of peripheral blood mononuclear cells (PBMC) and a sensitive HTA to measure relative virus production. A pairwise comparison was then performed to estimate the relative fitness of various non-syncytium-inducing/CCR5-tropic (NSI/R5) and syncytium-inducing/CXCR4-tropic (SI/X4) HIV-1 isolates. Four HIV-1 strains (two NSI/R5 and two SI/X4) with moderate ex vivo fitness were then selected as controls and competed against primary HIV-1 isolates from an HIV-infected Belgian cohort. HIV-1 isolates from long-term survivors (LTS) were outcompeted by control strains and were significantly less fit than HIV-1 isolates from patients with accelerated progression to AIDS (PRO). In addition, NSI/R5 HIV-1 isolates from PRO overgrew control SI/X4 strains, suggesting that not all SI/X4 HIV-1 isolates replicate more efficiently than all NSI/R5 isolates. Finally, there were strong, independent correlations between viral load and the total relative fitness values of HIV-1 isolates from PRO ($r = 0.84$, $P = 0.033$) and LTS ($r = 0.86$, $P = 0.028$). Separation of the PRO and LTS plots suggest that HIV-1 fitness together with viral load may be a strong predictor for the rate of disease progression.

In a human immunodeficiency virus (HIV)-infected individual, the rapid turnover (28, 53), high mutation rate, and high frequency of recombination result in a diverse population of HIV type 1 (HIV-1) quasispecies (52, 54). This extreme genetic diversity has distinct advantages for most RNA viruses, including (i) altered pathogenesis, (ii) evasion of host immune response and antiviral therapy, and (iii) development of new host (tissue, organ, or species) tropisms (20, 22). However, several host immunological and genetic factors rather than attributes of virus are generally described as having the greatest impact on HIV pathogenesis and progression to AIDS. For example, increased activity, maintenance, and HIV specificity of both CD4 and CD8 T-lymphocyte responses have been associated with slower disease progression (9, 10, 23, 37, 42, 43). It appears that survival of HIV-specific $CD4^+$ T cells after acute infection may provide the immune endurance necessary to control viremia (47). Although not directly related to HIV-specific immunity, chemotactic receptors (e.g., CCR5) and ligands (e.g., RANTES) also act as HIV-1 coreceptors and inhibitors, respectively (reviewed in reference 6). Polymorphisms or altered expression of the chemokines (e.g., RANTES) or chemokine receptor genes appear to influence disease progression (6).

While these genetic and immunological factors may influence pathogenesis, HIV remains the etiological agent of this disease. Biological phenotype/coreceptor usage of HIV is often associated with specific stages of disease, i.e., non-syncytium-inducing/CCR5-tropic (NSI/R5) isolates predominate during asymptomatic infections (50). Isolation of the faster-replicating syncytium-inducing/CXCR4-tropic (SI/X4) isolates late in disease had implied that a switch in biological phenotype/coreceptor usage was required for progression to AIDS (3, 7, 50). However, inconsistent isolation suggests that these SI/X4 isolates may be a result rather than a cause of late disease (3, 50). To date, HIV-1 load in plasma is the best viral correlate of disease progression (36). Highly active antiretroviral therapy (HAART), which reduces viral loads to undetectable levels, can also delay disease progression indefinitely or until the emergence of drug-resistant HIV variants. There is now mounting evidence that differences other than biological phenotype/coreceptor usage (e.g., variants of several HIV-1 genes) may contribute to both viral load and pathogenesis (16, 31). For example, a few long-term nonprogressors (LTNP) were shown to harbor HIV-1 strains with *nef* deletions (16, 31). These HIV-1 *nef* mutants display poor replication efficiency in tissue culture and have been characterized as less fit than wild-type HIV-1 isolates (15).

Fitness is a complex evolutionary term used to describe an

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organism's replicative adaptability in a given environment (20). Due to rapid replication and high mutation frequencies, RNA virus systems provide excellent models to test both fitness and evolutionary theories (11, 12, 20, 21, 29, 40, 55). In support of the red queen hypothesis, populations of virus quasispecies (e.g., foot-and-mouth disease virus, vesicular stomatitis virus, and HIV-1) in competition tend to gain fitness with each viral passage (12, 40). However, changes in environment and/or limits in population size may create bottlenecks and result in a reduced rate of fitness gains or even an overall loss in fitness (11, 20, 55). In these cases, the Muller's ratchet hypothesis suggests that an irreversible gain of deleterious mutations in limited populations will overwhelm the appearance of mutations improving fitness (11). Although in vitro competitions with HIV-1 and other RNA viruses may test an evolutionary theory, it is often difficult to apply these hypotheses to an actual infection. Immune response, antiviral therapy, and availability of target cells are only a few of the environmental factors that may affect viral replication, mutation frequency, and, as a result, fitness (21). In addition, the impact of HIV-1 fitness (e.g., a measure of replication efficiency) on disease progression is not well understood. Using a monoinfection assay with primary HIV-1 isolates, Blaak et al. (8) showed that some LTNP harbored NSI isolates with slow replication kinetics. Unfortunately, replication rates of HIV-1 in monoinfections are difficult to control, vary with different target cells, and must be restandardized when new isolates are tested (21, 29).

We have developed a competition assay for peripheral blood mononuclear cells (PBMC) to measure the ex vivo fitness of any primary HIV-1 isolates. Production of both HIV-1 isolates in the competition was analyzed by a heteroduplex tracking assay (HTA) and compared with initial inocula to calculate a relative fitness value for each isolate. Reproducible differences in HIV fitness were observed in pairwise competitions with several HIV-1 isolates and in PBMC from different donors. We then selected four HIV-1 isolates, two NSI/R5 and two SI/X4, for controlled competitions with primary HIV-1 isolates from three patients with accelerated progression to AIDS (PRO) and three characterized as long-term survivors (LTS). HIV-1 isolates from PRO outcompeted the control strains and were significantly more fit than the LTS-derived virus; which were overgrown by the controls. It is important to note that the term "more fit" in this context implies a greater ex vivo replication capacity in direct competition experiments. It does not infer that ex vivo fitness is the same as in vivo fitness, a very complex term encompassing both viral and host parameters. Regardless of the definition, ex vivo viral fitness in both PRO and LTS showed direct and independent correlations with viral load in these patients. These findings suggest that ex vivo fitness may influence and predict HIV-1 disease progression.

MATERIALS AND METHODS

Cells and viruses. PBMC from HIV-seronegative blood donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparin-treated venous blood. Prior to HIV-1 infection, the cells were stimulated with 2 μg of phytohemagglutinin (PHA; Gibco BRL) per ml for 3 to 4 days and maintained in RPMI 1640–2 mM L-glutamine medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro), 10 mM HEPES buffer (Cellgro), 1 ng of interleukin-2 (IL-2; Gibco, BRL)/ml, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (both from Cellgro). MT4 cells (obtained through the AIDS Research and Reference Reagent Program from Douglas Richman [32]) were cultured as described above but in the absence of IL-2 and PHA. All viral stocks were propagated and expanded in PHA-stimulated, IL-2-treated PBMC. Six SI HIV-1 isolates (laboratory-adapted strain B-HXB2 and five primary isolates [A-92UG029, D-92UG021, D-93UG067, E-CMU06, and F-93BR020]) and three NSI strains (laboratory-adapted strain B-BaL and two primary isolates [A-92RW009 and C-92BR025]) were obtained from the AIDS Research and Reference Reagent Program. For most strains listed above, the letter before the dash indicates the subtype of the viral envelope and precedes the year of isolation, country of origin, and strain number (e.g. A-92UG029 refers to a subtype A HIV-1 strain isolated in Uganda in 1992). Tissue culture dose for 50% infectivity, calculated by the Reed and Muench method (46) on the basis of reverse transcriptase (RT) activity in culture supernatants on day 8 of culture, was determined for each isolate in triplicate with serially diluted supernatants of each viral propagation. Titers were expressed as infectious units (IU) per milliliter.

HIV-1 isolates from the Belgian prospective cohort. HIV-1 primary isolates were selected from a well-characterized cohort of HIV-1-infected patients treated at the Institute of Tropical Medicine in Antwerp, Belgium. Samples from six HIV-1-infected individuals, initially diagnosed with HIV between 1985 and 1994 (24), were selected for this study. Based on $CD4^+$ cell count and clinical follow-up, two groups of three HIV-1 patients were defined: LTS (stable $CD4^+$ cell counts of >500 cells/μl for more than 5 years and naive to antiretroviral treatment) and PRO ($CD4^+$ cell counts decreasing to <200 cells/μl in 2 years or less and development of AIDS). Two blood samples were obtained from each patient in a period of 24 to 44 months. Two PRO patients (PRO2 and PRO3) had received antiretroviral treatment but only after the first sample collection (see Table 3). In this report, uppercase (e.g., PRO1) denotes patient identification, whereas lowercase denotes the virus derived from that patient at a particular time point (e.g., *pro*1A is the first virus isolated from patient PRO1). HIV-1 was immediately isolated by cocultivating HIV-infected PBMC with HIV-negative PBMC donors as previously described (39). The MT-2 assay was used to define viral phenotype (NSI or SI) (3). Coreceptor usage (CCR5 or CXCR4) was determined by exposing U87.CD4-CCR5 and U87.CD4-CXCR4 cells (kindly provided by H.-K. Deng and D. Littman [7] and obtained from the AIDS Research and Reference Reagent Program) to all HIV-1 patient isolates and assaying for infection as described elsewhere (51). An RT-PCR method was used to estimate viral RNA load in plasma (2). Briefly, HIV-1 particles were pelleted from 1 ml of plasma by centrifugation at 32,000 × *g* for 40 min. Viral RNA was extracted using a Qiagen RNeasy kit and QIAshredder spin columns (Qiagen). Complementary long terminal repeat DNA was reverse transcribed using primer AG4 (2) and Moloney murine leukemia virus RT (Gibco BRL) and then PCR amplified using unlabeled S1 primer and 5′-end-labeled γ-^{32}P-labeled A13 primer as described previously (2). As a positive control, 10-fold dilutions (10 to 10^8 copies) of HIV-1 pbs RNA (1) was RT-PCR amplified in parallel with the samples. Gels were dried, exposed to X-ray film (Kodak), and analyzed using a Molecular Dynamics PhosphorImager. Viral RNA loads quantified by this method were equal to those obtained using the Roche Amplicor assay (E. J. Arts, unpublished data). Finally, HIV-1 genetic subtype was determined by heteroduplex mobility analyses using *env* and *gag* fragments (L. Heyndrickx, unpublished data).

Growth competition assays. All dual infection/competition experiments were performed with PBMC from one donor (except where MT4 cells are indicated) on 24-well plates and in duplicate. As depicted in Fig. 1A, the dual infection/competition assay involved three separate dual infections with two HIV-1 isolates at different multiplicities of infection (MOIs; international units/cell, i.e., 0.1 IU of virus 1/cell plus 0.01 IU of virus 2/cell [Fig. 1A, well II], 0.1 IU of both viruses 1 and 2/cell [well III], and 0.01 IU of virus 1/cell plus 0.1 IU of virus 2/cell [well IV]). Uninfected culture in well VI was used as the HIV negative control, while wells I and V correspond to positive controls of only virus 1 and only virus 2, respectively. One-milliliter aliquots of these virus mixtures were incubated with 10^6 cells (PBMC or MT4) for 2 h at 37°C in 5% CO_2. Subsequently, the cells were washed three times with 1× phosphate-buffered saline and then resuspended in complete medium (10^6/ml). Cells were washed and fed with complete medium twice a week. New PHA–IL-2-prestimulated PBMC from the same donor or MT4 cells (5×10^5/well) were added weekly to replenish viable cells from cultures. Cell-free supernatants were assayed for RT activity every 5 days postinfection as previously described (51), and growth curves of each HIV-1 monoinfection (Fig. 1A, wells I and V) were constructed. Supernatants and two aliquots of cells were harvested at day 15, resuspended in dimethyl sulfoxide-fetal bovine serum, and then stored at −80°C for subsequent analysis.

PCR and RT-PCR of the HIV-1 *env* gene. For all competition experiments, proviral DNA was extracted from lysed PBMC or MT4 cells by using a QIAamp DNA blood kit (Qiagen). Viral RNA was purified from pelleted virus particles (cell-free supernatants centrifuged at 32,000 × *g* for 40 min) by using a Qiagen RNeasy kit and QIAshredder spin columns (Qiagen). Viral DNA, isolated from infected cells or reverse transcribed from viral RNA (i.e., using Moloney murine leukemia virus RT and the ED14 primer [18]), was PCR amplified using a set of external primers, envB (25)-ED14 (gp120-coding region of *env*, ~1.7 kb), followed by nested amplification using either the E80-E105 primer pair (48) (C2-C4 *env* region, 0.66 kb) or the E80-E125 primer pair (48) (C2-V3 *env* region, 0.48 kb) (Fig. 1B). Both external and nested PCRs were carried out in a 100-μl reaction mixture with defined cycling conditions (17). PCR-amplified products were isolated in agarose gels and then purified using a QIAquick PCR purification kit (Qiagen).

HTA for detection of two HIV-1 *env* fragments. Nested PCR products, C2-C4 or C2-V3 of the *env* gene, were analyzed by HTA (18, 19). The same genomic regions (C2-C4 and C2-V3) were PCR amplified from six subtype-specific HIV-1 strains (A-pRW20, B-pSF162, C-pMA959, D-pUG46, E-pTH22, and F-pBZ162) (17) for use as DNA probes. For this amplification, the E80 primer was radiolabeled using T4 polynucleotide kinase and 2 μCi of [γ-^{32}P]ATP. Radiolabeled PCR-amplified probes were separated on 1% agarose gels and then purified

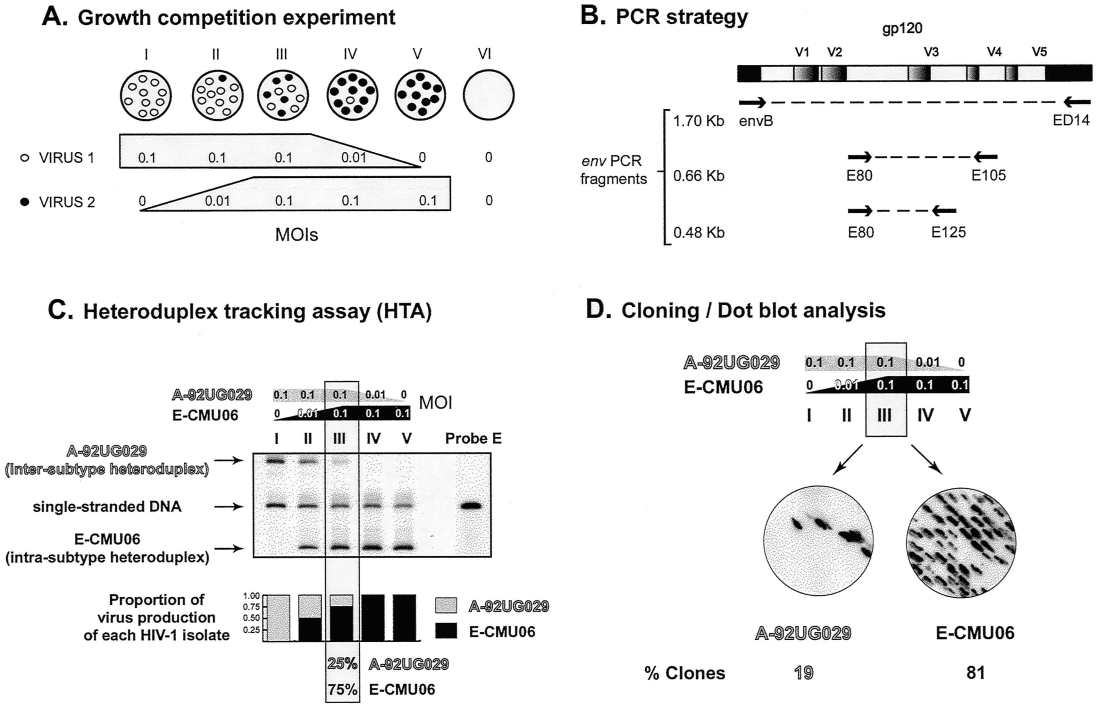


FIG. 1. Schematic representation of growth competition experiments and detection methods for dual infections. (A) Dual infections with a pair of HIV-1 isolates were performed at three different MOIs (wells II, III, and IV). Wells I and V correspond to positive controls for viruses 1 and 2. An uninfected culture (well VI) was used as a negative control. (B) Schema for PCR coamplification of two HIV-1 *env* fragments. Two *env* genomic fragments (C2-C4 and C2-V3 of the gp120-coding region) were PCR amplified from each dual (wells II, III, and IV) and single (wells I and V) HIV-1 infection, using first external primers (envB and ED14) and then one of two sets of nested primer pairs (E80-E105 or E80-E125). (C) Detection of two different HIV-1 isolates in growth competition experiments by HTA. Nested *env* PCR products from A-92UG029 and E-CMU06 competition were denatured and annealed to a subtype E probe (HIV-1 E-TH22). The percentage of each HIV-1 isolate in well III is shown below the autoradiograph. (D) Cloning/probe hybridization analysis of HIV-1 *env* fragments from a dual infection (well III). Ninety-six *env* bacterial colonies containing plasmid cloned from the 0.1:0.1-MOI dual infection (well III) were hybridized to subtype A or E *env*-specific oligonucleotides (4). The final percentage of each HIV-1 isolate in the dual infection is shown below the autoradiographs of hybridization blots.

using a QIAquick gel extraction kit (Qiagen). Reaction mixtures contained DNA annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA), 10 μl of unlabeled PCR-amplified DNA from the competition culture, and approximately 0.1 pmol of radioactive probe DNA. For each competition, two HTAs were performed using one of two probes specific for the subtype of each HIV-1 isolate in the dual infection. Reaction mixtures containing *env* DNA amplified from the competition and probe were denatured at 95°C for 3 min and then rapid annealed on wet ice. After 30 min on ice, the DNA heteroduplexes were resolved on Tris-borate-EDTA buffer–5% nondenaturing polyacrylamide gels (30:0.8 acrylamide-bisacrylamide) for 2.5 h at 200 V. Gels were dried, exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.), and scanned for analysis using 1D Image Analysis software (Kodak). Figure 1C shows an HTA of one competition (A-92UG029 plus E-CMU06) as an example of the HIV-1 intersubtype dual infection/competitions analyzed in this study.

An HTA control experiment was designed to validate the ability of HTA to detect and quantify each HIV-1 isolate in a competition assay. This first required cloning of *env* DNA from HIV-1 isolates A-92UG029, B-HXB2, D-92UG021, D-92UG067, E-CMU06, and F-93BR020. The full gp120-coding region of the *env* gene was PCR amplified as described above, but using the envA-envN (25) and envB-ED14 primer pairs for external and nested PCR amplifications, respectively. Nested PCR products (ca. 1.7 kb) were purified (QIAquick PCR purification kit; Qiagen) and cloned into the vector pCR II-TOPO (Invitrogen). The entire *env* gp120-coding region of each virus clone was sequenced as described below. Two *env* plasmids clones were mixed in PCR tubes at proportions equal to the initial inocula of virus used in the competitions. These in vitro mixtures were PCR amplified and subjected to HTA with two different clade-specific radiolabeled DNA probes as described above.

Cloning and probe hybridization for analysis of dual infections. Production of specific HIV-1 isolates in dual infections/competitions was measured by HTA (as described above) and using a cloning and probe hybridization technique. Briefly, *env* PCR fragments from the dual infection initially exposed to the same proportion of each virus (0.1 MOI [Fig. 1A, well III]) were cloned into pCR II-TOPO vector (Invitrogen). Ninety to 114 individual bacterial colonies containing an *env* plasmid from each competition were transferred to nylon membranes and lysed with 10% sodium dodecyl sulfate. Bacterial DNA covalently linked to the membrane was then denatured and hybridized to γ-^{32}P-labeled clade-specific *env* oligodeoxynucleotides as described elsewhere (4). Sequences and genomic positions of these primers are available upon request. Filters were autoradiographed and analyzed to calculate the final proportion of each HIV-1 isolate in the dual infection.

Estimation of viral fitness. Classic models for estimating replicative capacity or fitness of a virus are based in single competition experiments using equal MOIs of each virus. We have analyzed three dual infections in a competition experiment (MOI ratios of 10:1, 1:1, and 1:10, described above) (Fig. 1A) to provide a more accurate estimation of viral fitness. In our HIV-1 competition experiments, the final ratio of the two viruses produced from each of the three dual infections (wells II, III, and IV) was determined by HTA and compared to production in the monoinfections (wells I and V). Production of individual HIV-1 isolates in a dual infection (f_0) was divided by its initial proportion in the inoculum (i_0) to derive a single virus production (*ws*) value ($ws = f_0/i_0$).

A relative fitness (*w*) value for each virus was obtained from the average of the results of three independent dual infections (wells II, III, and IV). The ratio of relative fitness values of each HIV-1 variant in the competition (average of three dual infections) is a measure of the fitness difference (W_D) between both HIV-1 strains ($W_D = W_M/W_L$), where W_M and W_L correspond to the relative fitness of the more and less fit viruses, respectively. Our derivation of a fitness difference was compared to the selection coefficient, *s* (see Table 1, footnote *e*).

Nucleotide sequence analysis. The full gp120-coding region from HIV-1 isolates A-92UG029, B-HXB2, D-92UG021, D-93UG067, E-CMU06, and F-93BR020 was sequenced using the proprietary fmol method (Promega) as previously described (44). Primers used in the sequencing reactions (envB [25], ED5 [18], ED31 [18], E105 [48], E125 [48], and ED12 [18]) have been previously described. The V3 loop of the gp120-coding region of each of 12 HIV-1 isolates from Belgium was sequenced using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer). Nucleotide sequences were edited and translated by DNAsis version 2.6 software (Hitachi) and then aligned using the CLUSTAL X version 1.63b program.

Statistical methods. The statistical significance of differences between two different groups (e.g., $CD4^+$ cell counts or viral loads versus relative fitness HIV-1 values) was determined by the Mann-Whitney rank sum test. The Pearson product moment correlation coefficient was used to determine the strength of association or correlation between viral loads and HIV-1 relative fitness values.

Nucleotide sequence accession numbers. Nucleotide sequences reported in this study have been submitted to GenBank under the accession numbers given in parentheses: gp120-coding region of HIV-1 isolates A-92UG029 (AF205862), D-93UG067 (AF205863), and E-CMU06 (AF205864). Accession numbers of the nucleotide sequences encoding the V3 loop of HIV-1 isolates from the Belgian cohort are as follows: *lts*1a (AF205865), *lts*1b (AF205866), *lts*2a (AF205867), *lts*2b (AF205868), *lts*3a (AF205869), *lts*3b (AF205870), *pro*1a (AF205871), *pro*1b (AF205872), *pro*2a (AF205875), *pro*2b (AF205876), *pro*3a (AF205873), and *pro*3b (AF205874).

RESULTS

Detection and quantification of both HIV-1 strains in a dual infection/competition by HTA. Dual exposure and infection of host cells provides the best in vitro measure of relative HIV-1 fitness. For these growth competition experiments, we performed three dual infections with different inocula of both viruses (see Materials and Methods and Fig. 1A). Production of each isolate in the dual infection was impossible to detect using conventional assays (e.g., RT or p24 antigen capture assays), given that most HIV-1 isolates are indistinguishable from each other. Thus, we used a modified HTA to detect and quantify both HIV-1 isolates in the dual infections (Fig. 1C). To ensure ample separation of two heteroduplexes (representing the two HIV-1 isolates in a dual infection) or a nondenaturing polyacrylamide gel, we PCR amplified the most divergent HIV-1 gene, i.e., *env* (Fig. 1B). In addition, these HTAs utilized a probe sharing subtype identity with one isolate in the competition. This resulted in both intra- and intersubtype heteroduplexes migrating to different positions on the gel (Fig. 1C).

Two HIV-1 isolates in a mixture are easily identified by HTA. However, several controls were needed to validate this assay as an accurate method to quantify virus production. A competition between HIV-1 strains B-HXB2 and E-CMU06 was used for all of the following controls. To control for possible heterogeneity between the PCR primers and the target sequence in the heterogeneous *env* genes, two primer pairs (C2-V4 [0.66 kb] and C2-V3 [0.48 kb] [Fig. 1B]) were used to amplify different regions of *env*. Similar HIV *env* amplifications and subsequent detection by HTA were obtained with both primer pairs (Fig. 2A and B). C2-V3 *env* region, a 0.48-kb product, was amplified for all subsequent analyses. Next, we determined if sequence variation between isolates in the competition experiments could result in differential annealing to a probe. For these HTAs, a clade E or B *env* probe was denatured and annealed to PCR-amplified *env* products from the B-HXB2–E-CMU06 competition. As indicated by the mirror-like results in Fig. 2A and C, the clade E and B probes detected similar amounts of both isolates in the competition. In consideration of the similar levels of detection with two distinct probes, only HTA results with one probe are shown for all of the following competition experiments.

HTA is a sensitive assay to accurately detect and measure two HIV-1 isolates in a mixture. However, this technique was applied to HIV-1 DNA amplified from proviral DNA in the host cell. Thus, the actual production of virus progeny in the supernatant was compared to the amount of provirus in the host cells. Proviral DNA and viral RNA were PCR and RT-PCR amplified from B-HXB2–E-CMU06 competition and used in separate HTAs. Figures 2A and D show equivalent amounts of each isolate in the host cell (proviral DNA) and supernatant (viral RNA) 15 days postinfection. Comparison of proviral DNA and viral RNA analysis of several competitions showed similar results (data not shown). To avoid variability in the reverse transcription step, all subsequent HTA analyses were performed with provirus from the competitions.

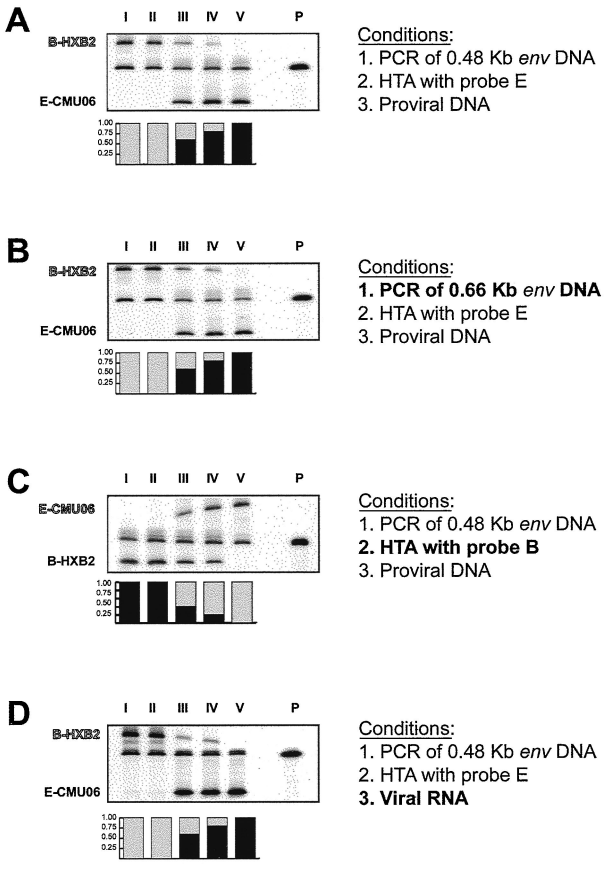


FIG. 2. Growth competition experiment with HIV-1 isolates B-HXB2 and E-CMU06 to validate the use of HTA as a dual virus detection method. (A) Standard conditions used in this study to analyze final proportions of two HIV-1 isolates from a dual infection: (i) proviral DNA was extracted from infected cells, (ii) a 0.48-kb *env* fragment (C2-V3) was PCR amplified by an external/nested technique, and (iii) HTA was performed with a subtype-specific probe (e.g., E-TH22). The same conditions were used for the subsequent controls, except for a larger (0.66-kb) *env* PCR fragment (C2-V4) (B), a probe specific to the subtype of the other HIV-1 isolate in the competition (B-SF162) (C), or viral RNA from supernatant culture (D). Roman numbers above the lanes correspond with the dual infections in Fig. 1A. P, lane containing only the subtype-specific probe.

HIV-1 competitions and estimation of HIV-1 fitness by HTA. Final ratios of two HIV-1 strains (Fig. 1A) were measured by HTA to derive relative fitness values for each HIV-1 isolate in a competition experiment (three dual infections). To further substantiate differences in viral fitness, we set up a series of HTA controls using proviral DNA constructs. *env* DNA clones from the six HIV-1 isolates were mixed in PCR tubes at quantities and ratios equivalent to MOIs used in competition experiments. This mixture was then subjected to the same PCR amplification and HTA analysis as outlined for the actual competition experiments. Figure 3 shows results of five HTA controls and five actual HIV-1 growth competition experiments. Final proportions of each HIV-1 strain were obtained by densitometry analysis of the strain-specific heteroduplex band (Fig. 3). In two of the viral competitions (Fig. 3B and C), one HIV-1 variant completely outcompeted its counterpart. This outgrowth of E-CMU06 over D-92UG021 and A-92UG029 over D-93UG067 was observed even with 10-fold excess of the weaker isolate in the initial inoculum (Fig. 3B and C, lane IV). Unlike these actual ex vivo competitions, the HTA control experiments using proviral *env* DNA constructs detected the

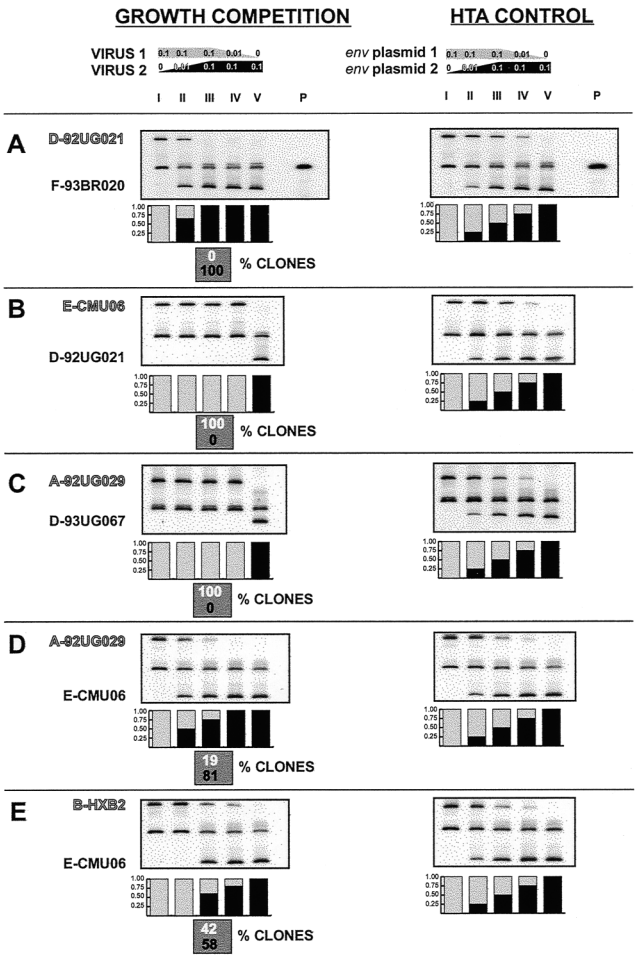


FIG. 3. Growth competition experiments and HTA controls with pairs of six primary HIV-1 isolates. Five pairs of two different SI/X4 HIV-1 isolates were used: (A) D-92UG021 plus F-93BR020, (B) D-92UG021 plus E-CMU06, (C) A-92UG029 plus D-93UG067, (D) A-92UG029 plus E-CMU06, and (E) B-HXB2 plus E-CMU06. The final percentage of each HIV-1 isolate in the dual infection was determined by HTA and by cloning/probe hybridization assays (indicated below well III). See legends to Fig. 1 and 2 for experimental details.

same ratio as was added to the initial mixture, a result expected in the actual competition if both isolates had equal fitness relative to each other.

Subtle differences were evident in the other three ex vivo competition experiments. HIV-1 isolate F-93BR020 had outcompeted D-92UG021 in all but one dual infection. Equal production of F-93BR020 and D-92UG021 (Fig. 3A, lane II) still suggests a competitive advantage for F-93BR020, considering that 10-fold less F-93BR020 was initially added to this dual infection. Competition experiments with A-92UG029 and E-CMU06 (Fig. 3D) and B-HXB2 and E-CMU06 (Fig. 3E) show even less competition and suggest only slight but reproducible differences in viral fitness. HTA controls using the A-92UG029 and E-CMU06 (Fig. 3D) or B-HXB2 and E-CMU06 (Fig. 3E) *env* plasmids show similar input and output ratios (as detected by HTA) and further confirm the sensitivity of this competition HTA experiment to determine HIV-1 fitness ex vivo.

As outlined in Fig. 1D, we compared the amount of dual isolate production obtained by HTA to that obtained by a cloning/probe hybridization assay (see Materials and Methods). Approximately 100 clones from 0.1:0.1-MOI dual infections (lane III) in each competition were screened by DNA hybridization (Fig. 1D). Using radiolabeled *env* probes specific for one or the other HIV-1 isolate in the dual infection (0.1:0.1 MOI) (lane III, Fig. 1D), we found that the proportion of clones hybridized to the two probes equaled the proportion of each isolate detected by HTA (shown below lane III in Fig. 3). For example, HTA detected 25% of A-92UG029 and 75% of E-CMU06 from 0.1:0.1-MOI dual infection, compared to 19 and 81% (respectively) detected by the cloning/hybridization assay (Fig. 3D). It is important to note that, using quantitative PCR with isolate-specific primers as well as the cloning/probe hybridization assay in *gag* and *env* genes, we determined that less than 1% of the total virus produced in 0.1:0.1-MOI dual infection was recombinant (M. E. Quiñones-Mateu and E. J. Arts, unpublished data). This value is consistent with other estimates of recombination in retrovirus coinfections (56). Due to this low frequency, *env* recombinants were not easily detected in the dual infections by HTA.

Deriving a numerical measure of relative fitness. Based on HTA results, we calculated relative fitness values for both isolates in each competition experiment (i.e., three dual infections). The relative fitness of each HIV-1 isolate is calculated as the average production in the three dual infections divided by the initial inoculum and is always relative to the value for the other HIV-1 isolate in these competitions. Fitness usually accounts for the relative number of progeny produced per cell per generation as well as possible host effects on virus production. There was insufficient virus production at days 5 and 9 to precisely determine the relative fitness of each isolate in a dual infection. The most accurate measure of individual virus production by HTA was obtained from a 15-day sample of each dual infection. This is, however, a snapshot of ongoing competitions. Prolonged incubations of these coinfections and a second passage in uninfected PBMC (>30 days) resulted in a complete outgrowth of the more fit isolate over the less fit (data not shown). In this study, a single passage with the potential for multiple viral replication cycles was sufficient for competition between two HIV-1 quasispecies populations (i.e., two primary HIV-1 isolates) and not between two single clones. Multiple passages would be required to measure outgrowth of single HIV-1 clones from the quasispecies population. This was not the intention of this study. An ex vivo fitness value of an HIV-1 isolate relative to another isolate was derived from the average virus production in three dual infections. Potential host effects on virus replication were not factored into this relative fitness value, because all competitions were performed with one batch of PBMC from a single HIV-negative donor. However, relative fitness values did not differ significantly in competitions performed with PBMC from different donors (see below).

Fitness difference in a competition between two HIV-1 isolates was obtained as described in Materials and Methods (Table 1). As expected, there were no significant differences in fitness (1.1- to 1.7-fold) in the HTA controls using the *env* plasmids. However, W_D values (2.4- to >100-fold) calculated from three dual infections (Table 1) did show a competitive advantage of one isolate over the other in the competition experiments (Fig. 3). In viral competitions where one HIV-1 isolate completely outcompeted the other (E-CMU06 > D-92UG021 and A-92UG029 > D-93UG067), W_D was greater than 100-fold (i.e., based on the detection limit for the weaker virus). Moderate W_D values (2.4- to 16.7-fold) were observed with the other competition experiments where differences in virus production were more subtle. Varying the proportions of each HIV-1 isolate in three dual infections did provide a better estimate of viral fitness than a single dual infection with equiv-

TABLE 1. Relative fitness values of HIV-1 primary isolates from dual competition experiments

Constructs used	Production of individual isolates relative to initial inoculum (f_0/i_0)[b]			Relative fitness[c]	Fitness difference[d] (fold)	Selection coefficient[e]
	II	III	IV			
Growth competition[a]						
HIV-1 isolates in dual competition						
D-92UG021	0.51 (0.46/0.9)	0.00 (0.00/0.5)	0.00 (0.00/0.1)	0.17		
F-93BR020	5.40 (0.54/0.1)	2.00 (1.00/0.5)	1.11 (1.00/0.9)	2.84	16.7	6.99
D-92UG021	0.00	0.00	0.00	0.00		
E-CMU06	10.0	2.00	1.11	4.37	>100	9.87
A-92UG029	1.11	2.00	10.0	4.37	>100	9.87
D-93UG067	0.00	0.00	0.00	0.00		
A-92UG029	0.53	0.50	0.00	0.34		
E-CMU06	5.20	1.50	1.11	2.60	7.6	6.97
B-HXB2	1.11	0.78	3.00	1.63	2.4	2.43
E-CMU06	0.00	1.22	0.78	0.66		
HTA control[f]						
HIV-1 *env* plasmids in a mixture						
D-92UG021	0.66	0.86	3.20	1.57		
F-93BR020	4.00	1.14	0.74	1.96	1.2	−0.98
D-92UG021	0.88	1.02	3.90	1.93	1.6	−0.84
E-CMU06	2.00	0.98	0.67	1.22		
A-92UG029	0.72	1.00	2.90	1.54		
D-93UG067	3.50	1.00	0.79	1.76	1.1	−0.96
A-92UG029	0.69	0.72	2.00	1.14		
E-CMU06	3.80	1.28	0.89	1.99	1.7	−0.65
B-HXB2	0.66	0.78	2.10	1.18		
E-CMU06	4.00	1.22	0.88	2.03	1.7	−0.74

[a] Growth competition experiments were performed with three dual infections (columns II, III, and IV). Two HIV-1 isolates were added at different MOI ratios (1:10, 1:1, and 10:1) as described in Materials and Methods (Fig. 1A).

[b] Single virus production (*ws*) corresponds to the final amount of each HIV-1 isolate produced from the dual infection (f_0) divided by the initial proportion of the virus in the inoculum (i_0). Two *ws* values represent the production of each isolate in that dual infection.

[c] Relative fitness (*w*) is the average of the single virus production values (*ws*) from each of the three dual infections [$w = (ws_{\mathrm{I}} + ws_{\mathrm{II}} + ws_{\mathrm{III}})/3$].

[d] Fitness difference (W_D) between HIV-1 isolates in the competition experiment was estimated by the ratio of relative fitness values ($W_D = W_M/W_L$), where W_M is the fitness of the most fit and W_L is the fitness of the least fit HIV-1 isolate in the competition. A greater than 100-fold difference corresponds to the detection limit of the less fit isolate.

[e] Selection coefficient (*s*) average (38). $s = \ln \dfrac{[p(t)/q(t)]}{[p(0)/q(0)]} / t$, where *p* and *q* are the proportions of the more and less fit variants in a competition experiment, respectively, *t* corresponds with the number of passages, and 0 corresponds with the initial proportion in the inoculum.

[f] Two *env* plasmid clones were mixed at proportions equal to the initial inocula of HIV-1 isolates used in the dual infections (Fig. 1A), as described in Materials and Methods.

alent inocula of both viruses, as is evident by comparing competitions in Fig. 3A and B. HIV-1 isolates F-93BR020 and E-CMU06 had completely outcompeted D-92UG021 at equal MOIs (Fig. 3A and B, lane III). When D-92UG021 was added in 10-fold excess over the competitor, a low level of D-92UG021 replication was evident in a dual infection with F-93BR020 (Fig. 3A) but completely absent in a dual infection with E-CMU06 (Fig. 3B). This difference in the 0.1:0.01 dual infections, not present in the 0.1:0.1 dual infections, was represented in W_D values derived from all three dual infections in a competition. There was a 16.7-fold W_D favoring F-93BR020 over D-92UG021 compared to a >100-fold W_D of E-CMU06 over D-92UG021 (Table 1). This derivation of W_D was also compared to the coefficient of selection (38). Assuming a single passage in the dual infections, there was a direct correlation between selection coefficients and W_D for each competition ($r = 0.94$, $P < 0.0001$, Pearson product moment correlation) (Table 1). A negative value for a selection coefficient (Table 1) suggests a lack of competition ($W_D \sim 1$) in HTA controls using *env* plasmids (Fig. 3).

All competitions in Fig. 3 used SI/X4 HIV-1 isolates and PBMC from the same donor. Relative fitness values were also calculated from pairwise viral competitions of three NSI/R5 HIV-1 isolates (A-92RW009, B-BaL, and C-92BR025) in PBMC (Fig. 4). HIV-1 B-BaL, a laboratory NSI isolate, was significantly more fit than the two primary NSI HIV-1 isolates, A-92RW009 and C-92BR025. A pairwise comparison of all HIV-1 primary isolates (three NSI/R5 and six SI/X4) in competition experiments revealed an ordered fitness of all HIV-1 isolates relative to each other. In general, the SI/X4 isolates were more fit and outcompeted the NSI/R5 isolates (data not shown). Based on this analysis, we selected two NSI/R5 and two SI/X4 isolates with moderate fitness (relative to all HIV-1 primary isolates) to be used as control strains in competition

TABLE 2. Comparison of relative fitness values obtained in a A-92UG029 versus E-CMU06 competition using different donor PBMC and a tumor T-cell line

Cell[a]	Production of individual isolates relative to initial inoculum[b]			Relative fitness[c]	Fitness difference[d] (fold)	Selection coefficient[e]
	II	III	IV			
MT4	0.48	0.47	0.00	0.32[f]		
	5.15	1.53	1.11	2.60[g]	8.1	7.54
$PBMC_1$	0.53	0.50	0.00	0.34		
	5.20	1.50	1.11	2.60	7.6	6.97
$PBMC_2$	0.50	0.45	0.00	0.32		
	5.12	1.51	1.11	2.89	9.1	8.01
$PBMC_3$	0.55	0.51	0.00	0.35		
	4.95	1.35	1.11	2.47	7.1	6.65

[a] Growth competition experiments were performed as described in Materials and Methods (Fig. 1A) and by adding the HIV-1 isolates A-92UG029 and E-CMU06 to MT4 cells or PBMC from three different HIV-seronegative donors ($PBMC_1$, $PBMC_2$, and $PBMC_3$).

[b–e] See Table 1, footnotes *b* to *e*.

[f] Relative fitness of A-92UG029 versus E-CMU06 in the competition.

[g] Relative fitness of E-CMU06 versus A-92UG06 in the competition.

assays with the Belgian patient-derived HIV-1 isolates (see below).

Finally, we investigated possible host PBMC effects on HIV-1 competitions. For these experiments, A-92UG029 and E-CMU06 (SI/X4 variants) were added to MT4 cells (a human T-cell leukemia virus type 1-transformed T-lymphocyte cell line) or PBMC from three different HIV-seronegative donors. Increased fitness of E-CMU06 over A-92UG029 was similar in all cells, i.e., 8.1-fold in the MT4 cells, 7.6-fold in $PBMC_1$, 9.1-fold in $PBMC_2$, and 7.1-fold in $PBMC_3$ (Table 2). Similar results were obtained when PBMC from three different donors were infected with two NSI/R5 strains (A-92RW009 and C-92BR025) (data not shown). However, this does not suggest that HIV-1 fitness is constant in all T-lymphocyte tumor cell lines or PBMC from all donors. Thus, only one preparation of PBMC from the same donor was used in all of the following competitions.

Clinical and virological data from the Belgian cohort. Two HIV-1 isolates were obtained from each of six patients (mean age of 36 years) over 24 to 44 months. Patients were followed for 4 to 11 years and characterized as LTS or PRO based on mean CD4 cell counts of 682 ± 175 or 319 ± 165 $CD4^+$ cells/ml ($P = 0.0043$, Mann-Whitney test), respectively (Table 3). It is important to note that the PRO cohort had drops in CD4 cell counts to <200/μl in less than 3 to 4 years but did not fit the strict definition of rapid progressors, i.e., patients with high viral burdens and AIDS within 2 to 3 years after seroconversion. Two of the infected patients with accelerated disease progression (PRO2 and PRO3) started antiretroviral therapy prior to isolation of the second virus sample, while the three LTS remained untreated. Virus phenotype was determined by the MT2 assay, coreceptor usage of $CD4^+$ U87 glioma cells, and *env* V3 sequence analysis (see Materials and Methods and Table 3). Both HIV-1 isolates propagated from LTS1, LTS2, PRO1, and PRO2 were NSI/R5, whereas LTS3 and PRO3 harbored SI/X4 strains. All HIV-1 isolates from this cohort were subtype B, except *pro*2A and *pro*2B (from patient PRO2), which were subtype A/E (Table 3).

TABLE 3. Clinical and virological parameters of LTS and PRO

Patient	Sex/infection route (dates [mo/yr] of follow-up)[a]	Age (yr) at yr of first $HIV\text{-}1^+$ test	CD4 count range[b] (cells/μl)	Sample-date[c] (mo/yr)	$CD4^+$ count (cells/μl)	Viral load (copies/ml)	Antiviral treatment[d] (date of initiation)	Virus phenotype[e]	Coreceptor usage[f]	V3 loop[g]		Subtype[h]	
										306	322	*gag*	*env*
LTS1	M/Hom (11/94–3/99)	40/1994	1,031–635	A-10/95	767	1,100	None	NSI	CCR5	G	E	B	B
				B-12/98	867	3,700	None	NSI	CCR5	G	E	B	B
LTS2	M/Hom (4/91–4/99)	23/1991	840–505	A-7/94	864	11,400	None	NSI	CCR5	S	E	B	B
				B-3/98	624	1,700	None	NSI	CCR5	S	E	B	B
LTS3	M/Hom (4/94–7/98)	30/1992	601–451	A-8/94	601	21,100	None	SI	CXCR4	S	E	B	B
				B-9/96	458	14,800	None	SI	CXCR4	S	H	B	B
PRO1	M/Hom (3/92–2/99)	42/1992	745–287	A-6/94	629	22,800	None	NSI	CCR5	S	E	B	B
				B-7/96	287	17,400	None	NSI	CCR5	S	E	B	B
PRO2	F/Het (3/94–1/99)	42/1994	319–71	A-3/94	142	4,200	None	NSI	CCR5	G	E	AE	ND
				B-7/96	71	2,400	AZT (7/95)	NSI	CCR5	S	G	AE	A/AE
PRO3	M/Hom (9/88–3/99)	41/1987	947–97	A-8/94	355	436,300	None	SI	CXCR4	S	R	B	B
				B-8/96	159	47,000	AZT + ddI, Saq + Nel (11/95, 5/96)	SI	CXCR4	S	R	B	B

[a] M, male; F, female; Hom, homosexual transmission; Het, heterosexual transmission.

[b] Obtained during dates of follow-up.

[c] A or B indicates one of two longitudinal blood samples from each patient taken at the date indicated. Samples A and B were used for $CD4^+$ cell counts, viral load, and HIV-1 propagations.

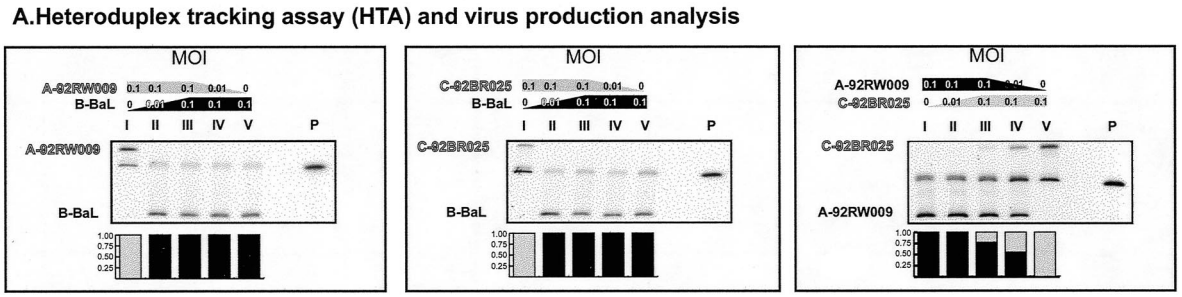
[d] AZT, 3′-azido-3′-deoxythymidine; ddI, 2′,3′-dideoxyinosine; Saq, saquinavir; Nel, nelfinavir.

[e] Determined by the MT2 assay (39).

[f] Determined in $CD4^+$ U87 brain glioma cells expressing either CCR5 or CXCR4 (51).

[g] Amino acids at positions 306 and 322 (numbering based on HIV-1 HXB2 strain) in the V3 loop of the *env* gene, i.e., residues associated with biological phenotype.

[h] HIV-1 subtyping was performed by heteroduplex mobility assay of the *env* (V3-V5 region) and *gag* genes (18) and confirmed by phylogenetic analysis of the V3 sequence. ND, not determined.



B. Fitness analysis

	w	W_D
A-92RW009	0.00	-
B-BaL	4.37	>100 - fold

	w	W_D
C-92BR025	0.00	-
B-BaL	4.37	>100 - fold

	w	W_D
C-92BR025	0.22	-
A-92RW009	2.94	13.3 - fold

FIG. 4. Three growth competition experiments with NSI/R5 HIV-1 isolates. Production of two NSI/R5 isolates (A-92RW009 plus B-BaL, B-BaL plus C-92BR025, and A-92RW009 plus C-92BR025) in each competition was measured by HTA (A). Relative fitness values were then derived from the three NSI/R5 dual infections from each competition (B) as described for Table 3.

HIV-1 fitness and disease progression. As described earlier, we have developed a rapid PCR/HTA method to measure relative fitness of two HIV-1 isolates in growth competition experiments. This dual infection/HTA technique was now used to compare ex vivo fitness of patient HIV-1 isolates to disease progression. However, it should be noted that ex vivo fitness derived from this assay is a measure of HIV-1 replication efficiency in competitions and is not always the same as in vivo fitness, a complex parameter accounting for host and viral factors affecting HIV-1 replication in vivo. Twelve HIV-1 isolates from the Belgian cohort (Table 3) were added to growth competition experiments along with each of four control primary HIV-1 isolates (SI/X4 strains A-92UG029 and E-CMU06; NSI/R5 strains A-92RW009 and C-92BR025). These control strains were selected based on moderate but different fitness values in pairwise competition experiments (Fig. 3 and 4, Table 1, and data not shown). Competition between each control strain and a Belgian HIV-1 isolate involved the same three dual infections and fitness calculation as described above. Figure 5 displays the fitness difference of LTS- and PRO-derived HIV-1 isolates relative to four HIV-1 control strains. Even though the equation for W_D ($W_D = W_M/W_L$) always produces a positive value, W_D was plotted as negative when the control strain was more fit than the patient isolate, and vice versa for a positive W_D.

Regardless of the viral phenotype (NSI or SI), HIV-1 iso-

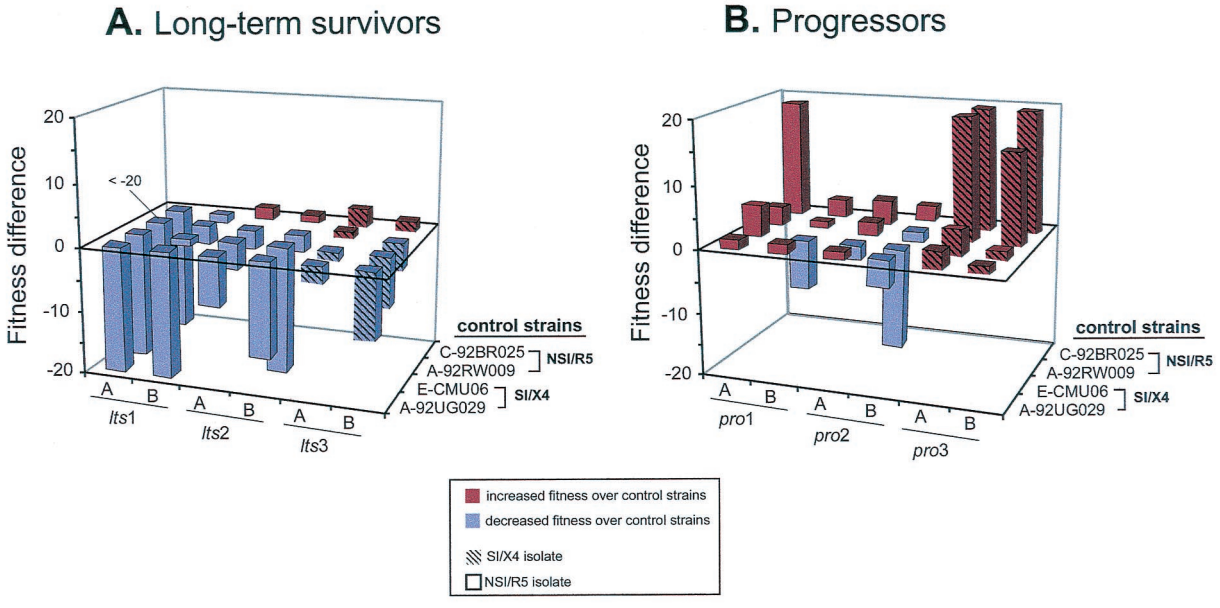


FIG. 5. The ex vivo fitness of HIV-1 isolated from LTS (A) and PRO (B) relative to four HIV-1 control strains. Within a 2- to 3-year period, two HIV-1 strains (designated A and B in the figure) were isolated from the three LTS and three PRO. A positive (red) or negative (blue) fitness difference corresponds to an *lts* or *pro* HIV-1 isolate being more or less fit than the HIV-1 control strain, respectively. Fitness differences were derived from the three dual infections in each competition with a control strain (two SI/X4 [A-92UG029 and E-CMU06] and two NSI/R5 [A-92RW009 and C-92BR025]). Limits of detection result in a maximum or minimum fitness difference of >100-fold. Dashed and solid bars correspond to competitions with SI/X4 and NSI/R5 patient HIV-1 isolates, respectively.

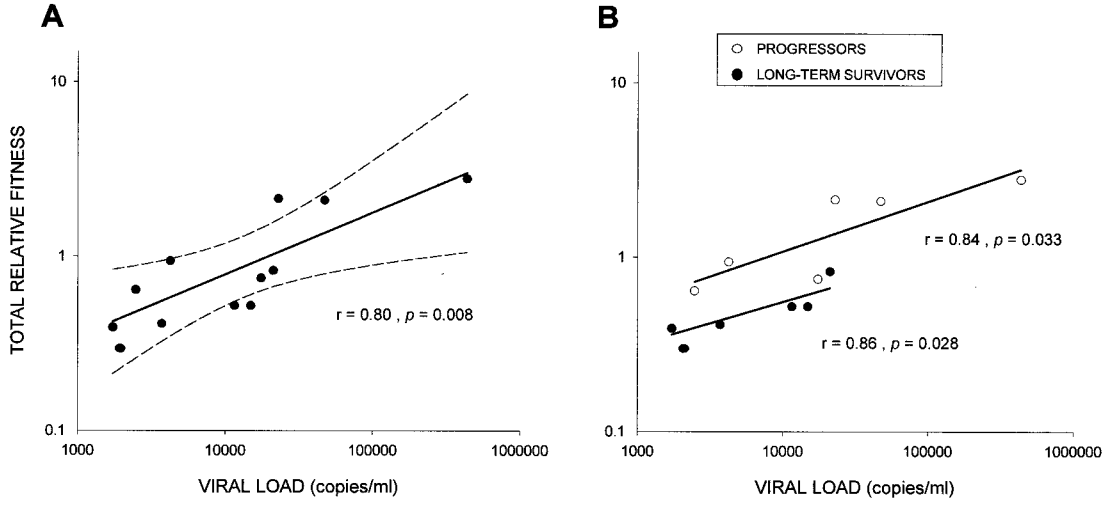


FIG. 6. Correlations between viral loads and the total relative fitness of HIV-1 isolates from infected individuals. Viral loads were plotted against the total relative fitness values of all HIV-1 isolates. Total relative fitness is the average of four relative fitness values, corresponding to the four competitions of each patient isolate with each of four control strains. Linear regression analysis (solid line) was then performed on the entire cohort (A), the LTS (B), and the PRO (B). Ninety-nine percent confidence curves (dashed lines) are shown in panel A.

lates from PRO had generally outcompeted the control strains in growth competition experiments, while the opposite was apparent with HIV-1 isolated from LTS. Relative fitness values versus the control strains were significantly greater for HIV-1 isolates from PRO than those from LTS (1.56 ± 1.24 and 0.44 ± 0.35, respectively; $P < 0.0001$, Mann-Whitney test). However, none of the *lts* or *pro* HIV-1 isolates showed significant differences in replication efficiencies during PBMC monoinfections. This difference in relative fitness derived from competitions was most dramatic when we compared isolates of the same phenotype from LTS or PRO. Although, *lts*1a and *pro*1a are both NSI/R5, *pro*1a had outcompeted the control strains and was >100-fold more fit than the *lts*1a HIV-1 isolate, which was overgrown by the same control strains (Fig. 5). Contrary to earlier findings, our data suggest that SI/X4 HIV-1 isolates are not always more fit or replicate more rapidly than NSI/R5 isolates. For examples, the HIV-1 SI/X4 isolate *lts*3b was outcompeted by the NSI/R5 control strain A-92RW009 (Fig. 5A), whereas the NSI/R5 isolate *pro*1a was slightly more fit than both the A-92UG029 and E-CMU06 SI/X4 isolates (Fig. 5B). These results suggest that SI/X4 HIV-1 isolates from LTS are significantly less fit than those from PRO. Thus, not all SI/X4 isolates outcompete NSI/R5 isolates, or vice versa.

Correlating ex vivo fitness with viral load. Although ex vivo HIV-1 fitness was significantly less in LTS than in PRO ($P < 0.0001$, Mann-Whitney), this correlation with types of disease progressors (e.g., LTS and PRO) is quite broad by definition and does not provide a direct comparison with various clinical determinants of HIV-1 pathogenesis (e.g., CD4 cell counts and viral load). Each relative fitness value of all Belgian HIV-1 isolates was plotted against RNA viral loads or CD4 cell counts (obtained from the blood samples used for the virus isolation). There was no correlation between relative fitness and CD4 cell counts ($r = -0.42$, $P > 0.05$, Pearson product moment). However, a positive correlation was observed between relative fitness of each patient isolate (derived from each competition with a control strain) and the respective viral loads ($r = 0.58$, $P < 0.01$ with A-92UG029; $r = 0.60$, $P < 0.01$ with E-CMU06; $r = 0.77$, $P < 0.001$ with A-92RW009; and $r = 0.76$, $P < 0.01$ with C-92BR025). This positive correlation was greatest ($r = 0.80$, $P = 0.008$, Pearson product moment correlation) when viral loads are compared with the total relative fitness values (i.e., average of relative fitness values, derived from a competition between a patient isolate and each of A-92UG029, E-CMU06, A-92RW009, and C-92BR025) (Fig. 6A). Use of a total fitness value implies that the control strains had the same order of relative fitness values when competing against all patient isolates. In general, (i) the SI/X4 control strains provided stronger competition against all patient isolates than the NSI/R5 control strains and (ii) the order of control strain fitness did not vary in pairwise competitions of control strains (Fig. 3 and 4, Table 1, and data not shown). Finally, the correlation with viral load was higher with the total fitness values than with any individual fitness value (see above). Thus, competitions with several control strains and inclusion of each relative fitness value into a total fitness value may provide a better estimate of the actual ex vivo fitness.

Positive correlations were also observed between total fitness and viral load when LTS and PRO groups were analyzed separately: $r = 0.86$, $P = 0.028$ for the LTS group, and $r = 0.84$, $P = 0.033$ for the PRO group (Pearson product moment correlation) (Fig. 6B). Higher P values were due to smaller numbers in LTS and PRO groups than in the total population. Considering parallel slopes and no intersection between the extrapolated regressions for LTS and PRO groups, ex vivo HIV-1 fitness values together with viral loads may be a strong and significant predictor of slow versus rapid progression to AIDS.

DISCUSSION

Despite numerous studies on fitness of RNA viruses, few have focused on (i) fitness of HIV-1 and (ii) the impact of ex vivo HIV-1 fitness on disease. Most studies on HIV-1 fitness have compared the replication efficiencies of drug-resistant and wild-type clones (14, 27, 35, 49) or have estimated in vivo fitness in different HIV-1 subpopulations (26). We have performed competition infections with pairs of primary HIV-1 isolates and developed a relatively rapid detection technique (dual infection/HTA) to estimate HIV-1 fitness. Multiple tests were performed to validate the use of HTA in HIV-1 fitness

studies. These include PCR amplification of two different *env* regions and hybridization to two different radiolabeled probes to rule out isolate-specific amplification or isolate-specific hybridization. All controls confirmed that HTA was an appropriate and sensitive technique to quantify two viruses in a dual infection. The HTA assay did not detect any recombined *env* fragments in these dual infections. Frequency of HIV-1 recombination in the *env* and *gag* genes during these dual infections was approximately 1% of the total virus production (Quiñones-Mateu and Arts, unpublished). Unlike other techniques (e.g., phenotypic, cloning/hybridization, or cloning/sequencing assays), HTA can also be used without prior knowledge of HIV-1 sequence or biological phenotype. Using this assay system, it was apparent that HIV-1 isolates from PRO were significantly more fit than HIV-1 isolates from LTS. In addition, the relative fitness values from both PRO and LTS showed strong, independent correlations with viral load. Details of these findings are discussed below.

Fitness is a measure of an organism's adaptation to a given environment. For any environment, the amount of each variant in a population is directly dependent on its ability to compete with other quasispecies (11, 12, 21, 29, 40, 55). Rapid replication of RNA viruses (e.g., foot-and-mouth disease virus, vesicular stomatitis virus, or HIV-1) coupled with high mutation rates permits a continual selection of the most fit variants from an expanding pool of quasispecies. Competitions between two highly related quasispecies may require several passages to detect a difference in fitness (12, 29). For example, zidovudine-resistant clones with single substitutions (e.g., M41L or K70R in the RT-coding region) from the parental wild-type strain required high virus production and several passages in a tumor cell line to discern a fitness difference from the wild type (27). In contrast, competitions between divergent clones or quasispecies populations require fewer replication cycles and show greater differences in relative fitness (20, 29). All of our competitions involved primary HIV-1 isolates, each of which is a population of quasispecies isolated and propagated from a patient sample.

To reduce selection in tissue culture, we performed single viral expansions with all primary isolates from the NIH-AR-RRP-WHO repository or from the Belgian cohort. Although PBMC from a single donor and blood draw were used in all competitions, we still compared results of competition between SI/X4 subtype E (E-CMU06) and A (A-92UG029) strains in a tumor T-lymphocyte cell line (MT4) and in PBMC from three different donors. Interestingly, little or no change was observed in the relative fitness of each isolate in these competitions. This does not imply that all HIV-1 isolates infect and replicate to equal extents in PBMC from all donors. However, most host factors that cause variations in replication efficiencies may not be isolate specific, aside from the obvious restrictions due to coreceptor usage. Using equal inocula of two divergent HIV-1 isolates, 15 days (or up to 15 viral replication cycles) was often sufficient for some isolates to completely overgrow the other in a dual infection of PBMC. As a consequence, an increased inoculum of the less fit isolate over the more fit isolate (MOIs of 10:1) provided a better estimate of relative fitness. In a pairwise comparison, two SI/X4 HIV-1 subtype D isolates appeared less fit than the SI/X4 isolates of other subtypes. However, several competitions with multiple subtype D isolates are necessary to suggest a lower ex vivo fitness of this subtype than of other HIV-1 group M subtypes. Interestingly, two laboratory isolates, the SI/X4 B-HXB2 and NSI/R5 B-BaL strains, were significantly more fit than any primary HIV-1 isolates of the same biological phenotype. These findings suggest that multiple passages in tissue culture results a strong adaptation of laboratory clones.

Since the early 1990s (33), a number of reports have described a privileged subset of HIV-infected individuals with stable CD4 cell counts (>500/μl), a strong HIV-specific immune response, and low viral loads (9, 10, 23, 37, 42, 43, 47). Several factors, as opposed to a dominant host or viral trait, appear to influence and/or predict slow disease progression. These include HIV-specific immune responses (e.g., $CD4^+$ T-cell proliferative response, cytotoxic T-lymphocyte response, and possibly humoral response) (9, 10, 23, 37, 42, 43, 47), host genetic background or polymorphisms (e.g., HLA I types or polymorphisms in the chemokine or chemokine receptor alleles) (6, 30), and viral factors (e.g., deletions in the *nef* gene) (3, 8, 16, 31, 50). Apart from the inconsistent observation that a switch from a slow NSI/R5 to a faster-replicating SI/X4 phenotype precedes AIDS (3, 7, 9, 42, 50), few studies have compared HIV-1 fitness to disease progression (8). In 1995, an LTNP in Australia was shown to harbor HIV-1 with a *nef* deletion (16). Interestingly, a similar *nef*-deleted HIV-1 clone was engineered as an attenuated strain for vaccine development (15). However, the vast majority of LTNP do not harbor a *nef*-deficient strain or another isolate with a known defect in any HIV-1 gene (13, 45). Blaak et al. (8) recently reported that LTNP harbor NSI/R5 HIV-1 isolates significantly less fit than HIV-1 of the same phenotype in typical progressors. Although this result predicts a correlation between viral fitness and disease progression, a significant difference between HIV monoinfections is necessary to discern variations in replication efficiency. In contrast, based on growth curves of each HIV-1 monoinfection, all of the primary isolates used in this study showed the same replication kinetics and viral production levels and did not contain *nef* mutations associated with altered replication (data not shown). Considering that (i) some LTNP or LTS harbor HIV-1 isolates with poor replicative ability and (ii) this competition assay could detect discrete variations in HIV-1 fitness, we determined the relative fitness of two HIV-1 isolates from each of three LTS and three PRO. There was a sharp contrast between the relative fitness values of LTS- and PRO-derived HIV-1 isolates; i.e., PRO HIV-1 isolates competed more efficiently with control strains and were more fit than LTS-derived HIV-1 isolates. These findings suggest that HIV-1 fitness may be a correlate and/or effector of disease progression in HIV-infected individuals.

In general, two HIV-1 phenotypes are associated with different stages of disease. Infection was established by an NSI/R5 isolate even though most infected individuals were exposed to a mixture of both NSI/R5 and SI/X4 strains (57). NSI/R5 strains dominate throughout asymptomatic disease but are replaced by SI/X4 isolates prior to AIDS in at least half of HIV-infected individuals (3, 9, 42). Over the years, few have challenged the concept that SI/X4 (or T-cell line-tropic) strains replicate more efficiently in PBMC monoinfections than NSI/R5 (or macrophagetropic) HIV-1 isolates. In this study, a pairwise competition experiment with several NSI/R5 and SI/X4 primary isolates and laboratory strains (BaL and HXB2) supports the notion that SI/X4 isolates replicate more efficiently than NSI/R5 strains. However, this inference may not hold true for HIV-infected individuals showing atypical progression. Two SI/X4 isolates from patient LTS3 were outcompeted by the NSI/R5 control strain, C-92BR025. Three NSI/R5 isolates (*pro*1a, *pro*1b, and *pro*2a) from two patients with accelerated disease progression had overgrown an SI/X4 control isolate, A-92UG029. Thus, the correlation between fitness and disease progression (e.g., viral load) was independent of viral phenotype.

To date, the HIV-1 load in plasma is the best predictor of disease progression (9, 36, 42). Factors such as HAART and strong HIV-specific immune responses can effectively reduce viral loads to undetectable levels and delay disease progression. We observed a positive correlation between the relative fitness values of all HIV-1 isolates and the corresponding viral loads. In addition to an increase in ex vivo fitness over the course of infection, the relative HIV-1 fitness values from PRO and LTS showed even stronger, independent correlations with viral load. Thus, it appears that the ex vivo fitness value together with viral load may be a strong and important predictor of progression to AIDS. We are now compiling a large repository of HIV-1 isolates from untreated individuals for a more comprehensive analysis of the impact of HIV-1 fitness on disease progression and to assess any relationship with viral load and HIV-specific immune responses. HIV-specific $CD4^+$ T-cell proliferative responses showed a similar but inverse correlation with viral loads (47). Recent studies suggest that a strong HIV-specific cell (cytotoxic T-lymphocyte or T-helper)-mediated response after acute infection and maintained throughout disease may reduce the rate of progression (41, 47). Furthermore, treatment with HAART during acute infection is thought to preserve the $CD4^+$ T cells and support this HIV-specific immune response (5). Based on these findings and our results on ex vivo fitness, we and others (34) have proposed that fitness of the infecting HIV-1 isolate may be paramount to the subsequent disease and status of HIV-specific immune response.

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