

Brief Report

A PATIENT WITH HIV-1
SUPERINFECTION

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THE detection of an increasing number of circulating recombinant strains of human immunodeficiency virus type 1 (HIV-1) indicates that genetic recombination can occur in cells infected with two strains of HIV-1.^{1,2} Coinfection with two circulating strains of HIV-1 has been detected in a few subjects in communities where HIV-1 infection is endemic.³ Coinfection may result from exposure to a second virus either shortly after the initial infection or during the course of established HIV-1 infection; the latter circumstance is called superinfection.

Most viral infections induce lifelong immunity, but reinfection with respiratory viruses such as respiratory syncytial virus is common, most likely because immunity becomes nonprotective or fades. It is thought that HIV-1 superinfection is a rare event¹⁻⁶ and that it is prevented by previous viral exposure through a phenomenon called superinfection immunity.⁵ However, HIV-1 superinfection has been induced experimentally in chimpanzees.⁷ In this animal model and in superinfection induced with the simian immunodeficiency virus in macaques, the second infection produces a slower deterioration in immunity than does the initial infection,⁷⁻⁹ and there is more efficient control of viremia. In this article, we report a case of HIV-1 superinfection.

CASE REPORT

In November 1998, a 38-year-old man presented with an acute retroviral syndrome. Anti-HIV-1 antibodies were undetectable, the level of p24 antigen was greater than 100 pg per milliliter, the plasma level of HIV-1 RNA was 805,000 copies per milliliter, and the CD4 cell count was 684 per cubic millimeter. Sequences of the HIV-1 reverse transcriptase and protease genes revealed no mutations associated with drug resistance and identified the HIV-1 as

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subtype AE. For years, the patient had had sexual contacts with multiple unknown male partners. He enrolled in the QUEST trial¹⁰ in November 1998 and received highly active antiretroviral treatment (HAART) with zidovudine, lamivudine, abacavir, and amprenavir for 27 months. From month 21 to month 27 he participated in a vaccination trial and was randomly assigned to receive ALVAC vector vCPI452.¹¹

Six weeks after the initiation of HAART, the plasma level of HIV-1 RNA declined to 1000 copies per milliliter. Treatment was then interrupted for six weeks because of toxic effects on the liver. After HAART was resumed, the HIV-1 RNA level decreased rapidly, to less than 50 copies per milliliter (Fig. 1). After vaccination, HAART was again interrupted (on January 21, 2001) as part of the vaccine research protocol. In February 2001, the patient's plasma HIV-1 RNA level rose to 80,000 copies per milliliter (the first rebound) and then decreased to 21,000 copies per milliliter. A rapid increase in the HIV-1 RNA level was next observed on April 10 (the second rebound), and for the next four months the level fluctuated between 200,000 and 400,000 copies per milliliter. The patient's symptoms (transient fatigue and fever) were mild, and he declined to resume HAART during this period. His history revealed that he had had several unprotected sexual contacts in Brazil three weeks before the second rebound of viremia. Four months after the second rebound, however, HAART was resumed, and the plasma level of HIV-1 RNA rapidly decreased. Treatment was again interrupted after an increase in the alanine aminotransferase level to 800 U per liter, as compared with a level of 200 U per liter before HAART. At this time, serologic data and quantification of hepatitis C virus (HCV) RNA (Fig. 1) documented an acute HCV infection. The increase in alanine aminotransferase was attributed both to HCV infection and to drug-induced toxic effects. Treatment with pegylated interferon and ribavirin was followed by clearance of HCV RNA (to less than 500 copies per milliliter) within two months.

METHODS

Assays

The levels of HIV-1 and HCV RNA in the plasma were quantitated (Amplicor HIV-1 Monitor Test, version 1.5, Roche Diagnostics), and serologic analyses, including analysis of p24 antigen, were performed with enzyme immunoassays (Abbott). The following steps were performed as previously described^{12,13}: purification of plasma RNA and proviral DNA, population sequencing of HIV-1 reverse transcriptase and protease and of the *gag* p17 and *env* C2V3 gene regions, and analyses of sequences.

Subtype-Specific Polymerase Chain Reaction

Primers were selected according to the patient's subtype AE and subtype B protease and reverse-transcriptase sequences, as follows: AE sense primer (position 2289 to 2312, based on the subtype B reference genome HXB2; GenBank accession number, K03455), 5'ACAGTAAAAATAGGAGGACAG; B sense primer, 5'ATAAAGGTAGGGGGCAATTAAAG; AE antisense primer (position 2786 to 2806), 5'TCCTGAGTTCCTTTTATTGAGC; and B antisense primer (position 2687 to 2712), 5'GATTTTCAGGCCCAATTTTGTAAATT. The polymerase chain reaction (PCR) consisted of 40 amplification cycles at 95°C for 20 seconds and 65°C for 30 seconds. For each PCR, 100 µl of RNA was extracted from plasma.

In mixing experiments, the subtype-specific primers allowed the detection of approximately 20 copies of the homologous subtype among 250,000 copies of the heterologous subtype and did not amplify the heterologous subtype. Successive plasma samples were amplified by both primer pairs within the same experiment. Twenty microliters of the amplicons was placed on a nylon membrane, which was hybridized with digoxigenin-end-labeled probe (2431 to 2451, 5'ATTAAAGCCAGGAATGGATGG) and revealed with

the use of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (DIG High Prime DNA labeling and Detection Starter Kit II reagents, Roche Diagnostics) as recommended by the manufacturer.

HIV-1 Proviral DNA PCR

PCR was performed with 1 µg of DNA purified from peripheral-blood mononuclear cells by nested PCR. The first reaction consisted of 40 amplification cycles at 95°C for 20 seconds and 50°C for 30 seconds with the use of sense and antisense primers (positions 1627 to 1655 and 2791 to 2812 of HXB2). Five percent of the amplicons were introduced into the nested PCR with the use of subtype-specific primers as described above. In mixing experiments, the subtype-specific primers allowed the detection of approximately 10 copies of the homologous proviral DNA subtype among 1000 copies of the heterologous subtype and did not amplify the heterologous subtype. Twenty microliters of the amplicons was subjected to electrophoresis on 2 percent agarose gel and stained with ethidium bromide.

Isolation and Growth of Subtypes AE and B in Vitro

Stored peripheral-blood mononuclear cells collected at the time of acute infection and one month after the second rebound of viremia after the discontinuation of HAART were depleted of CD8

T cells by beads coated with anti-CD8 (Dynabeads, Dynal), cocultivated with phytohemagglutinin-stimulated CD8-depleted peripheral-blood mononuclear cells from a single uninfected blood donor. Culture supernatants were collected during viral exponential growth, which was assessed by quantification of p24 antigen in culture supernatant (detection limit, 3 pg per milliliter). New cultures of CD8-depleted peripheral-blood mononuclear cells (2×10⁶ cells) were inoculated in duplicate with 20 pg and 100 pg of p24 antigen recovered from the primary cultures containing the AE and B subtypes, respectively. Viral replication was monitored by quantification of p24 antigen in the culture supernatants.

Interferon-γ CD8 Enzyme-Linked Immunospot Assay

Peptides (8- to 15-mers) were synthesized according to the patient's available subtype AE and B sequences and his HLA subtypes (HLA-A*01/02 and HLA-B*51/57). CD8 enzyme-linked immunospot assays (Elispot) were performed as previously reported.¹⁴ We also used an algorithm to generate a score that would predict the degree of binding between a peptide and an HLA molecule.¹⁴

RESULTS

The evolution of HIV-1 viremia and CD4 cell counts is shown in Figure 1. Successive serologic analy-

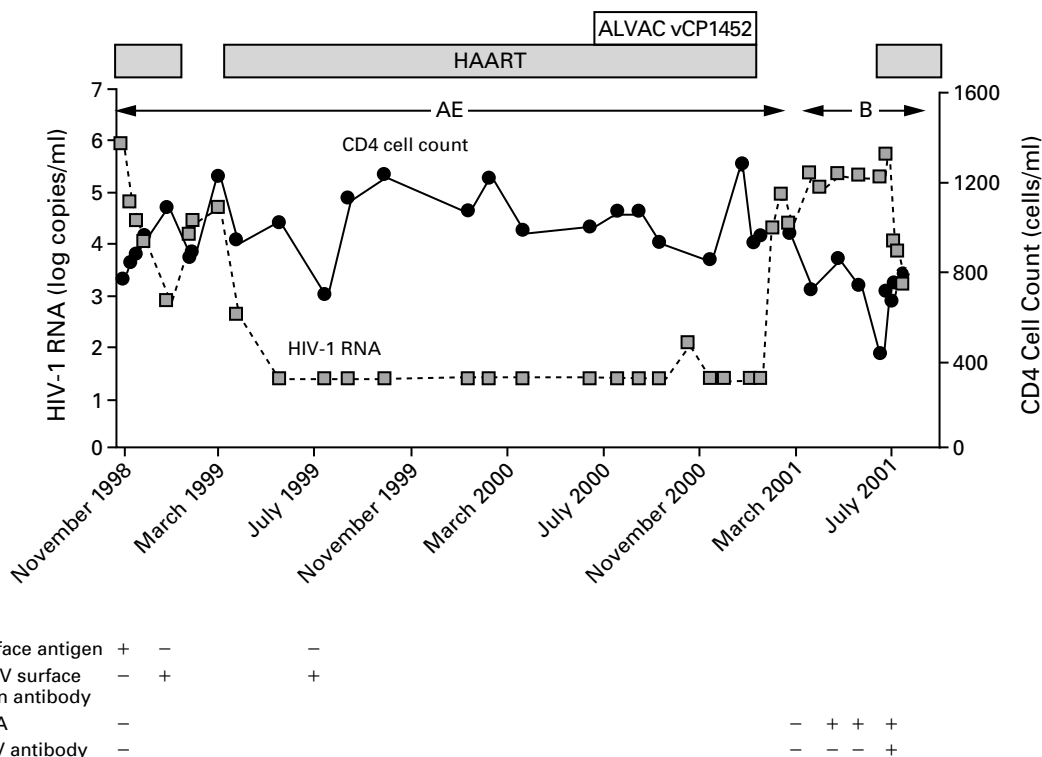


Figure 1. HIV-1 RNA Levels and CD4 Cell Counts during Acute HIV-1 Infection and 31 Months of Follow-up.

The limit of detection of HIV-1 RNA was 10 copies per milliliter. The horizontal bars show the periods during which the patient received highly active antiretroviral therapy (HAART) and the period of vaccination with ALVAC vCP1452, and the horizontal arrows show the change from subtype AE to subtype B. Also shown are the results of serologic tests for hepatitis B virus (HBV) and hepatitis C virus (HCV) and the qualitative results of analysis of plasma HCV RNA. Plus signs denote a positive result, and minus signs a negative result. The first sample positive for HCV RNA contained 103,000 copies per milliliter and was of genotype 3a.

ses for hepatitis B virus (HBV) and HCV as well as quantification of HCV RNA indicate that the patient was coinfecting with HBV and HIV-1 in October or November 1998 and was infected with HCV in about March 2001. Sequencing of the reverse transcriptase and protease genes in three successive plasma samples obtained at the time of acute infection, in a DNA sample obtained in January 2001, and in a plasma sample obtained at the time of the first rebound (in February 2001) identified the HIV-1 subtype AE in all instances (data not shown). Sequencing of the *gag* p17 and *env* C2V3 regions performed during the same period also indicated that the AE subtype was present. In contrast, sequencing of reverse transcriptase, protease,

gag p17, and *env* C2V3 from plasma collected in April, May, and November 2001 indicated the presence of the HIV-1 subtype B (GenBank accession numbers: subtype AE, AY122635-40, and subtype B, AF517666-72). Figure 2 shows the results of the phylogenetic analysis of the *env* C2V3 sequences. The subtype B sequences in our patient from the end of April 2001 to April 2002 segregated with subtype B sequences from Brazil (BR.U16219, BR.U08800, and BR.U08799), with a bootstrap value of 86 percent.

To enhance the sensitivity of the detection of HIV-1 species, we performed plasma PCR with subtype B- and AE-specific primers. The only detectable virus circulating in plasma during the acute HIV-1 infection

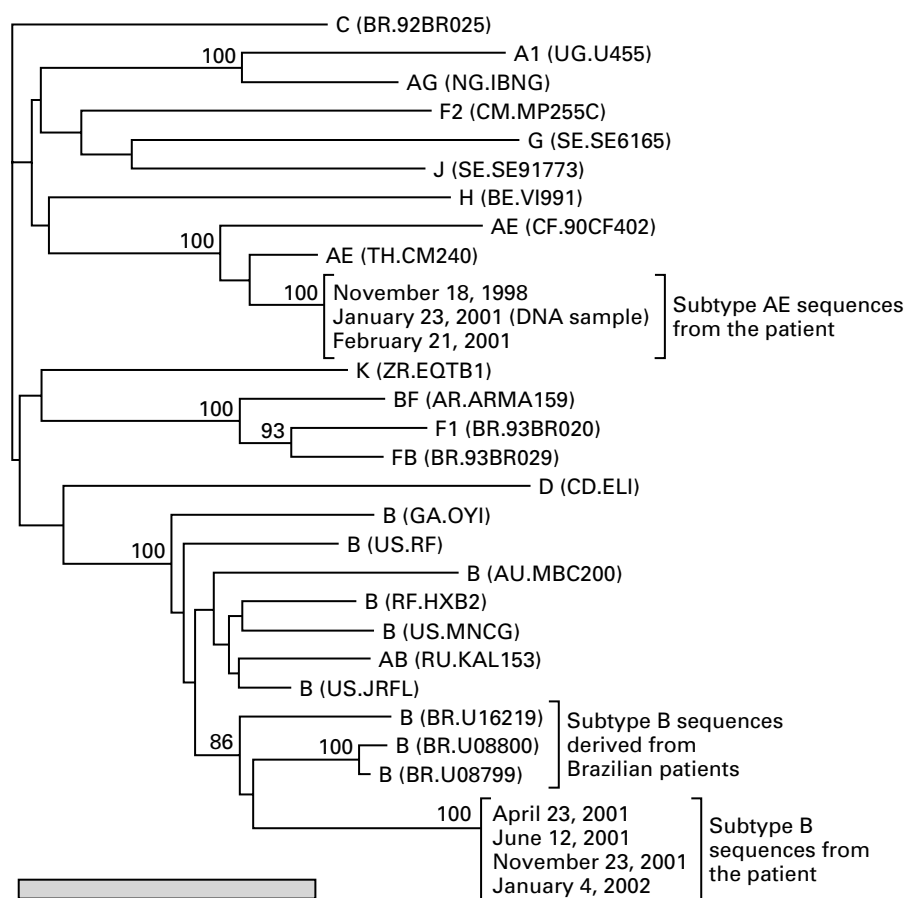


Figure 2. Phylogenetic Analysis of *env* C2V3 Sequences.

Phylogenetic analysis was performed according to the Fitch–Margoliash method (with Phylogeny Inference Package software)¹⁵ with selected reference sequences, including subtype B sequences from Brazil and the patient's sequences derived from plasma samples (obtained November 18, 1998; February 21, April 23, June 12, and November 23, 2001; and January 4, 2002) and from a proviral DNA sample (obtained January 23, 2001). Letters outside parentheses are HIV-1 subtypes; codes inside parentheses refer to the country of origin and the number of the sequence according to the HIV Los Alamos data base (<http://hiv-web.lanl.gov>).² Bootstrap values greater than 80 percent are indicated. The shaded bar indicates a genetic distance of 0.1 (10 percent divergence of nucleotide sequences).

from November 1998 to March 2001 was of the AE subtype (Fig. 3A). In April 2001, during the second rebound of viremia, amplicons from both the AE subtype (weak staining) and B subtype (predominant staining) were detected. In subsequent plasma samples, the B subtype was predominant, with intermittent traces of the AE subtype. Only the AE-specific primers amplified proviral DNA purified from peripheral-blood mononuclear cells collected in November 1998 and March 2001, whereas both AE- and B-spe-

cific primers amplified DNA collected in April 2001 (Fig. 3B). Three months later, only B subtype amplicons were detectable.

The responses of CD8 cells to the patient's AE- and B-specific epitopes were investigated with the use of enzyme-linked immunospot assays. Only cells directed against AE-specific epitope SLYNTVATL (denoted with single-letter codes for amino acids) were detected between November 1998 and March 2001 (from 595 to 727 spot-forming cells per 10^6 peripher-

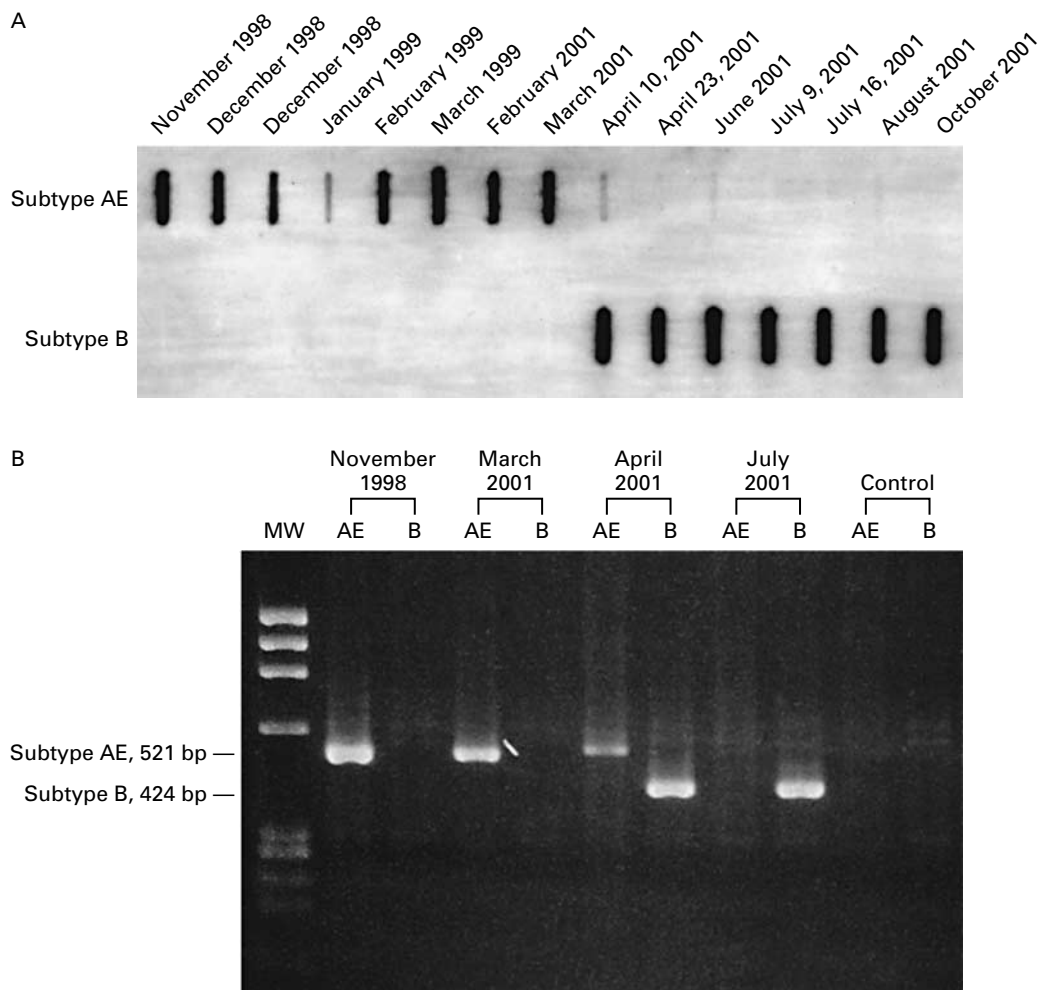


Figure 3. HIV-1 Subtype-Specific Analysis of Plasma RNA and Proviral DNA Amplified by the Polymerase Chain Reaction. Panel A shows the results of immunoblot analysis, with chemiluminescence of amplicons derived from plasma HIV-1 RNA collected during the acute HIV-1 infection up to October 2001. The top row shows samples amplified by subtype AE-specific primers, and the bottom row shows samples amplified by subtype B-specific primers. Panel B shows the results of electrophoresis, with ethidium bromide staining of amplicons derived from HIV-1 proviral DNA. Proviral DNA from peripheral-blood mononuclear cells was amplified by nested polymerase chain reaction. Subtype AE and subtype B amplicons had the expected molecular size, expressed in base pairs. The control corresponds to DNA purified from an uninfected, healthy blood donor. The first column contains molecular weight (MW) markers (ϕ X174 restriction-fragment DNA purified with *Hae*III).

al-blood mononuclear cells). They markedly decreased in number after the switch to the B subtype (epitope SLFNTI AVL) in April 2001 (100 spot-forming cells per 10⁶ peripheral-blood mononuclear cells) (Table 1). This B sequence, which contained mutations affecting the HLA-A2 binding score (which was 157 for the AE peptide and 75 for the B peptide), was not recognized at any time. In addition, 10 other reverse-transcriptase peptides corresponding to AE or B sequences were tested but were not identified at any time.

We assessed the growth of subtype AE and B isolates in cultures with inocula of 20 pg and 100 pg of p24 antigen. The release of p24 antigen in culture supernatant was detected on day 3 with both the 100-pg and 20-pg inocula of B isolate, whereas with the subtype AE isolate, p24 antigen was detected only on day 7 (100-pg inoculum) and day 9 (20-pg inoculum).

DISCUSSION

The patient we describe had HIV-1 seroconversion in November 1998 as a result of infection with HIV-1 subtype AE; the infection was successfully treated with HAART. After the interruption of HAART at the end of January 2001, subtype AE viremia rebounded (in February); the viremia then declined during the following two weeks before a second rebound, at which time the HIV-1 RNA level plateaued at 400,000 copies per milliliter. This second rebound involved HIV-1 subtype B, which rapidly replaced subtype AE. This subtype B virus segregated with isolates from Brazil, a finding consistent with the patient's recent travel and sexual exposures in that country.

This observation provides strong evidence of HIV-1 superinfection. To rule out coinfection, we attempted to amplify subtype B by means of subtype-specific PCR. We failed to detect subtype B in plasma RNA or in proviral DNA samples obtained up until the second viremia rebound. In addition, the subtype B isolate had a higher replicative capacity in vitro than the subtype AE isolate and, once detected, rapidly replaced subtype AE in vivo.

Strong, HIV-1-specific CD8-cell responses were directed against a single AE-derived epitope during the period of infection with subtype AE. None of the subtype B epitopes derived from the patient's sequences were recognized at any time during either subtype AE or subtype B infection. The drop in subtype AE CD8 cells at the time of the switch in subtypes could be attributed to the sequence variation in the B epitope that affected HLA-binding capacity. That the inhibition of subtype AE replication was due to an immune response to ALVAC epitopes after immunization cannot be ruled out, since the p17 peptide SLYNTVATL in the patient's AE was identical to the "subtype B" *gag* sequence inserted into ALVAC.¹¹

The biologic course after HIV-1 subtype B superinfection was characterized by the persistence of high plasma levels of HIV-1 RNA and a loss of 300 CD4 cells per cubic millimeter within the four months after the emergence of subtype B. The subtype B superinfection led to rapid progression of disease,¹⁶ in contrast to the pattern of disease observed after experimental superinfection in monkeys.⁷⁻⁹ The early initiation of HAART in our patient may have limited his

TABLE 1. RESPONSES OF CD8 CELLS TO PEPTIDE-SPECIFIC EPITOPES.

PEPTIDE*	SEQUENCE†	PERIOD‡	FREQUENCY OF PEPTIDE-SPECIFIC T CELLS§			
			NOVEMBER 20, 1998 (SUBTYPE AE)	FEBRUARY 12, 2001 (SUBTYPE AE)	MARCH 15, 2001 (SUBTYPE AE)	MAY 15, 2001 (SUBTYPE B)
spot-forming cells per 10 ⁶ PBMC						
<i>gag</i> 77-85						
Subtype AE	SLYNTVATL	1998-2000	727	700	595	100
Subtype B	--F--I-V-	May 2001	0	0	5	0
Reverse transcriptase 33-41						
Subtype AE	ALTEICKEM	1998-2000	0	20	45	0
Subtype B	--V---T-L	May 2001	0	26	40	0

*The HLA restriction element is HLA-A2.

†The peptide sequence shown (with one-letter codes for amino acids) is that of the virus isolated from the patient. Only the amino acids differing between both sequences are shown.

‡The periods during which samples yielding the viruses with sequences shown in the previous column were obtained are listed.

§The threshold of positivity of this immunospot assay is 50 spot-forming cells per 10⁶ peripheral-blood mononuclear cells (PBMC) after the subtraction of background.

exposure to HIV-1 antigens and his HIV-1-specific immune responses. In North America and western Europe, subtype B predominates and diversifies rapidly within individual patients,¹⁷ rendering the detection of superinfection especially difficult; the frequency of superinfection might thus be underestimated.

Our data indicate that natural infection does not necessarily induce cross-clade protection. There should be close monitoring of circulating HIV-1 strains in the context of vaccine development. Our observations also support the use of safe-sex precautions even among HIV-1-infected persons.

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