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Intersubtype Human Immunodeficiency Virus Type 1 Superinfection following Seroconversion to Primary Infection in Two Injection Drug Users

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In this study, we describe two cases of human immunodeficiency virus type 1 (HIV-1) intersubtype superinfection with CRF01_AE and subtype B strains, which occurred in two injection drug users participating in a prospective cohort study in Bangkok, Thailand. In both cases, the superinfecting strain was detected by molecular and serologic analyses several weeks after complete seroconversion to the primary infection with a strain belonging to a different subtype. Superinfection occurred despite specific T-cell and humoral antibody responses to the primary virus. In both cases, cross-subtype immune responses were limited or absent prior to the second infection. These data show that, in some individuals, the quality and quantity of the immune response elicited by primary HIV-1 infection may not protect against superinfection. This finding has important implications for vaccine design. HIV-1 vaccines, at a minimum, will need to include potent, broadly protective, conserved immunogens derived from several group M subtypes.

Whether human immunodeficiency virus type 1 (HIV-1) superinfection occurs in humans is not well established. Superinfection is defined as the reinfection of an individual, after a primary HIV-1 infection, with a heterologous strain belonging to the same subtype as the primary strain or to a different one. Given the broad spectrum of genetically distinct subtypes of HIV-1 (31), it is important from the standpoint of vaccine development to determine if the immune responses generated during a primary infection will recognize and clear a challenge virus. A few studies have demonstrated HIV-1 coinfection, which is the simultaneous transmission of genetically distinct HIV-1 variants belonging to the same subtype (intrasubtype) (10, 32) or different subtypes (intersubtype) (3, 7, 18, 23, 30). The routes of transmission for these coinfections were vertical transmission, sexual transmission between spouses, and blood transfusion (10, 17, 23). However, most multiple HIV-1 subtype infections have been identified from cross-sectional studies or from convenience samples with little or no information regarding the source of the virus and timing of transmission. Therefore, it is unknown if any of these multiple infections were superinfections. In humans, a preliminary report had suggested that intrasubtype B superinfection had occurred, resulting in accelerated disease progression (J. B. Angel, S. Kravcik, E. Balaskas, P. Yen, A. D. Badley, D. W. Cameron, and Y.-W. Hu, Abstr. 7th Conf. Retrovir. Opportunistic Infect., abstr. LB2, 2000). However, the full details of this case have not been published and it is unclear whether the strain causing the primary infection, the superinfecting strain, or a potential recombinant had resulted in rapid progression to AIDS. Thus, the pathogenic consequences of superinfection, if any, are also unclear.

Among several possible strategies to detect potential intersubtype superinfection, one strategy would be to prospectively monitor a cohort of uninfected individuals at high risk for HIV infection within a population in which two or more HIV-1 subtypes are prevalent, coupled with long-term follow-up of any HIV seroconverters. These parameters were available in a collaborative study of a prospective cohort of seronegative injection drug users (IDUs) in Bangkok, Thailand, which is managed by the Bangkok Metropolitan Administration (BMA) (20, 37). As described previously, HIV-1 incidence in this population is high (6%) and individuals are at high risk for infections from two HIV-1 group M variants: CRF01_AE and subtype B (9, 16, 20, 37). The HIV-1 epidemic in this IDU cohort was characterized by a period of high incidence during the third and fourth quarters of 1996, when the incidence rose to 11.4 and 8.5 cases per 100 person-years, respectively (14). The majority (81%) of HIV-1 strains from seroconverters during this period of high incidence formed several strong phylogenetic associations, indicating HIV transmission networks among the IDUs (24). The presence of both CRF01_AE and subtype B strains, the high incidence of HIV-1 infection, and the high-risk behavior among Bangkok IDUs increased the likelihood of dual subtype infections and the subsequent emergence of novel recombinant strains. HIV-1 CRF01_AE strains are found predominantly among persons in southeast Asia. These strains are considered to be recombinant genomes which display subtype A-like *gag*, *pol*, and *env* gp41 regions,

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whereas the *env*-gp120 region has been characterized as belonging to an HIV-1 subtype E lineage whose parental strain has not been identified (8, 13). The objectives of this study were to determine if superinfections had occurred among the seroconverters and to estimate the time interval between the primary infection and superinfection.

MATERIALS AND METHODS

Study setup and follow-up. The BMA maintains a municipal drug treatment program in Bangkok, Thailand, through which approximately 10,000 drug users seek treatment annually. A total of 1,209 HIV-1 negative IDUs were enrolled in a prospective cohort study from May 1995 through December 1996, as described previously (37). All seronegative cohort participants were tested at 4-month intervals for HIV antibodies by enzyme immunoassay (EIA) (20). Participants who tested positive at a scheduled 4-monthly visit, designated the first positive (FP) time point, were offered voluntary enrollment in a follow-up study of seroconverters. Additional blood samples were collected, usually 3 to 6 weeks after the FP time point, at a visit designated T0 (15, 37). Following T0, blood was collected approximately 1 month later (time point designated T1) and then at 4-month intervals thereafter (designated collection time points T4, T8, T12, etc.). The scheduled 4-month visit prior to the FP time point at which the participant was negative by EIA was designated the last negative (LN) time point. In this study set, we used samples collected from 130 seroconverters. Only serum samples were collected from the LN and FP time points. HIV-1 sequence analysis at T0 revealed that 103 of 130 (79.2%) of the seroconverters were infected with HIV-1 CRF01_AE strains and 27 of 130 (20.7%) were infected with HIV-1 subtype B strains (35).

Cross-sectional screening. RNA derived from serum samples collected at FP and DNA from peripheral blood mononuclear cells (PBMC) collected at T12 were screened by restriction fragment length polymorphism (RFLP) of the *pro* gene region for evidence of mixed (subtypes A + B) restriction patterns. A dual-subtype pattern at T12 coupled with a single-subtype pattern at FP would suggest that superinfection with a heterologous strain had occurred in the interval between FP and T12.

RFLP. Since HIV-1 CRF01_AE strains are a mosaic of genomic regions belonging to subtypes A and CRF01_AE, in which the *pro* gene belongs to subtype A, *Alu*I restriction patterns of the *pro* gene were used to screen for the presence of a mixed A + B pattern (28), which would be indicative of both CRF01_AE and subtype B strains in a sample. Samples that gave a dual (A + B) *Alu*I restriction profile were selected for cloning and phylogenetic analysis of the *pro* gene to confirm the presence of both subtype variants.

Subtype-specific PCR. Several sets of nested primers designed to be specific for CRF01_AE and subtype B gp120 sequences were developed. After extensive testing of sensitivity and specificity of the various primer combinations, one set of nested primers for CRF01_AE and subtype B was chosen for further use.

The nested primers used in this study amplify a 687-bp product within gp120 (*env*). The primers, along with their coordinates (in parentheses) relative to the HXB2 genome (GenBank accession no. K03455 and M38432) are as follows. For CRF01_AE: outer forward SSE1, 5′-TGA AGG AGA CAC AGA TGA ATT GGC CAA-3′ (6232 to 6258); outer reverse SSE8, 5′-TTC CTG TAA TAT TTG ATA CAC AAT-3′ (7555 to 7578); nested forward SSE5, 5′-AAC TGT TCT TTT AAT ATG ACC ACA GAA C-3′ (6689 to 6717); and nested reverse SSE6, 5′-ATA GAA AAA TTC CCC TCT ACA ATT AAA ATG A-3′ (7346 to 7376). For subtype B: outer forward SSB1, 5′-TGA MGG GGA TCA GGA AGA ATT ATC AGC-3′ (6232 to 6258); outer reverse SSB8, 5′-(A/G)CC CTG TAA TAT TTG ATG AAC ATC-3′ (7555 to 7578); nested forward SSB5, 5′-AAC TGC TCT TTT AAT ATC ACC ACA AGC A-3′ (6689 to 6717); and nested reverse SSB6, 5′-GTA GAA AAA TTC CCC TCC ACA ATT AAA ACT G-3′ (7346 to 7376). The subtype B and CRF01_AE primer sets were tested for sensitivity by using serial dilutions of known copy numbers of CRF01_AE and subtype B gp120 clones developed previously (27). The CRF01_AE nested primers were consistently able to detect a minimum of 100 input copies of subtype E clones (data not shown). The subtype B primers were consistently able to detect a minimum of 1 input copy of subtype B gp120 clones (data not shown). The CRF01_AE primers did not amplify 500, 10^3, and 10^4 input copies of subtype B clones, demonstrating their specificity for CRF01_AE sequences. The subtype B primers did not amplify 500, 10^3, and 10^4 input copies of CRF01_AE clones, demonstrating their specificity for subtype B sequences (data not shown).

DNA cloning and phylogenetic analysis. PCR products were purified and cloned in the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.). Multiple sequence alignment was done by using PILEUP in the GCG Wisconsin Package version 10.0 (Genetics Computer Group, Madison, Wis.). Alignments were manually edited and gap stripped. Neighbor-joining trees were constructed with 500 bootstrap replicates by using both MEGA (Molecular Evolutionary Analysis) version 1.02 (21) and ClustalW version 1.7 (36) programs. These neighbor-joining trees incorporated standard sequences representing *gag* and *env* HIV-1 subtypes A, B, D, and E, which were obtained from the HIV database at http://www.hiv-web.lanl.gov.

Laboratory testing for clinical parameters of infection. Viral load determinations were performed by using the Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Branchburg, N.J.). The lower limit of quantitation was 400 RNA copies/ml. Lymphocyte immunophenotyping was done on fresh EDTA-anticoagulated venous samples following the first seropositive visit with the FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) using a standard six-tube, two-color monoclonal antibody panel (Becton Dickinson). A V3 peptide-based EIA was used to measure serum antibody reactivities to HIV-1 CRF01_AE and subtype B-specific V3 peptides (26, 38).

IFN-γ ELISPOT. An enzyme-linked immunospot (ELISPOT) assay for the detection of gamma interferon (IFN-γ) release by stimulated cells was conducted by previously published methods (19). T-cell responses were evaluated using "in-well" infections of subjects' PBMCs with recombinant vaccinia virus constructs (22). Specifically, recombinant vaccinia viruses containing HIV-1 CRF01_AE and subtype A gene regions were used. The wild-type New York City Board of Health (NYCBH) strain constructs used were vT27 (*env* from CRF01_AE strain CM243), which was a gift from Gail Mazzara, Therion Biologics Corp., Cambridge, Mass.; vT142 (*gag* from subtype A strain 90CF402.1); vT141 (*nef* from 90CF402.1); vT143 (*pol* from subtype A strain 92UG037.1); and NYCBH. For evaluating HIV-1 subtype B-specific T-cell responses, Western Reserve (WR) vaccinia virus constructs containing subtype B gene regions were used. The WR constructs used were vP1174 (*env* from HIV-1MN); VP 1287 (*gag* from HIV-1 IIIB); vP 1218 (*nef* from HIV-1 MN); and vP 1288 (*pol* from HIV-1 IIIB) and WR (wild-type WR strain) from the National Institutes of Health, AIDS Research and Reference Reagents Program, Bethesda, Md.

RESULTS

Two cases of HIV-1 superinfection were found in this study. These will be referred to as case 1 and case 2.

Case 1: follow-up history and detection of superinfection. Case 1 was a 30-year-old IDU who enrolled in the initial prospective cohort study of HIV-1-seronegative IDUs in June 1996. At the time of enrollment, she reported that her last drug injection had taken place 10 days prior to her enrollment visit and that she had shared injection equipment with one other person. She denied any sexual relationships at that time and at all subsequent study interviews. Four months following enrollment, case 1 tested positive on HIV-1 EIA (FP). There was no behavioral information available until May 1997 when case 1 reported for her T4 visit.

Case 1 was identified by cross-sectional screening of the seroconverter sample set consisting of 130 cell-free RNA samples at FP and the corresponding 130 PBMC-DNA lysates at T12. As mentioned earlier, CRF01_AE strains possess an HIV-1 subtype A protease (*pro*) gene region. Therefore, RFLP of the *pro* gene, which can discriminate between HIV-1 subtypes A and B (17, 28, 30), was used for cross-sectional screening. A single subtype (A) pattern was detected at the FP collection point; however, a dual subtype (A + B) restriction pattern was detected at T12 (approximately 1 year following seroconversion) (RFLP data not shown). Additional RFLP analysis of *pro* from both cell-free RNA and PBMC-DNA from all collection time points between FP and T12 detected only HIV-1 subtype A at FP and T0, and both HIV-1 subtypes A and B from T1 through T12 (RFLP not shown). The accuracy of the results obtained by RFLP was tested by cloning and sequence analysis of the *pro* gene. Only subtype A clones were

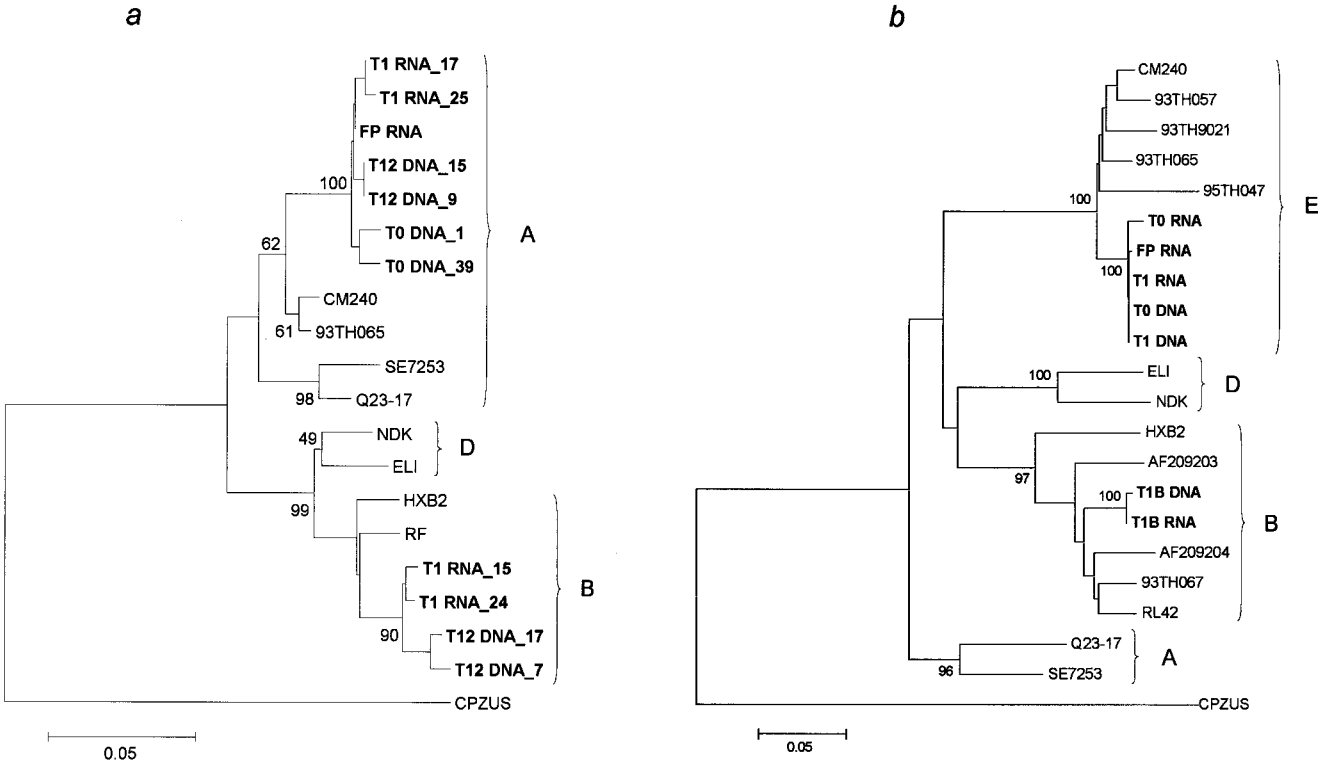


FIG. 1. Phylogenetic analysis of HIV-1 sequences from case 1 (bold). (a) Representative sequences from *pro* subclones (297 bp) from FP, T0, T1, and T12 collection time points. (b) gp120 (*env*) population sequences (618 bp) obtained from PCR products generated by subtype-specific primers. Sequences generated during this study were obtained from the FP, T0, T1, and T12 collection time points. The source of the sequence (PBMC-DNA lysate or cell-free RNA) is indicated after each collection time when appropriate. Subclones are indicated by an underscore. Standard sequences representing HIV-1 subtypes were obtained from the HIV database at http://www.hiv-web.lanl.gov. HIV-1 subtypes are indicated to the right of the trees, and bootstrap values are indicated to the left of the branch nodes. The trees are unrooted, and branch lengths are proportional to the numbers of molecular changes between individual operational taxonomic units.

obtained at FP and T0; however, both subtype A and subtype B clones were obtained at T1 and T12 (Fig. 1a). Twenty-seven *pro* subclones were examined at T1, and 18 of 27 (67%) were subtype A and 9 of 27 (33%) were subtype B. In order to reconfirm that T1 was the earliest time at which a dual subtype infection could be detected, differential subtype amplification was performed with the CRF01_AE- and subtype B-specific gp120 region primers (Fig. 1b). The dual subtype infection at T1 was detected in both cell-free RNA and HIV-infected PBMC (Fig. 1b). Multiple attempts to amplify the subtype B gp120 from FP and T0 with the subtype B-specific primers were negative.

These data indicated that primary infection in case 1 had occurred with a strain of CRF01_AE, as evidenced by detection at the earliest available collection times, i.e., FP and T0. This was followed by detection of a subtype B strain along with the CRF01_AE strain, first at T1 and then at subsequent follow-up times. Superinfection most likely occurred in the interval between T0 (24 December 1996) and T1 (17 January 1997).

Case 2: follow-up history and detection of superinfection. Case 2 was a 32-year-old IDU when he enrolled in November 1995. He reported very frequent drug injection and sharing of injection equipment. During the entire period of follow-up, case 2 reported that he was living with his common-law wife and denied any casual sex partners. At the T8 visit, he reported having used heroin since his last visit (T4) and also reported having injected 3 days prior to his T8 visit. He also reported sharing injection equipment with three drug-using partners.

Case 2 was identified in a subset of seroconverters for whom samples beyond T12 were being characterized. We used generic primers, detailed previously (34), to amplify the C2-V4 region of *env* from T32. Population sequencing and phylogenetic analysis of the C2-V4 PCR product derived from T32 plasma in three different experiments yielded a CRF01_AE classification. However, previously we had identified a subtype B strain by C2-V4 population sequence at T0 for case 2 (35). The sequence chromatogram of the T32 sequence revealed two peaks at most of the informative positions (base positions different between consensus CRF01_AE and B C2-V4 sequences; data not shown). The dual-subtype infection was confirmed by isolating C2-V4 region subclones belonging to both HIV-1 CRF01_AE and subtype B from the T32 sample (representative T32 subclones in Fig. 2). Of 11 C2-V4 subclones which were examined, 8 of 11 (73%) were CRF01_AE and 3 of 11 (27%) were subtype B. To estimate when, in the interval between T0 and T32, the dual-subtype infection had occurred, subtype-specific PCR was performed by using cell-free RNA and PBMC-DNA lysate samples. HIV-1 subtype B was detected in cell-free RNA and infected cells for all the time points, T0 through T32, and representative collection times were used for phylogenetic analysis (Fig. 2). In contrast, we did not detect CRF01_AE from T0 through T4. We first detected

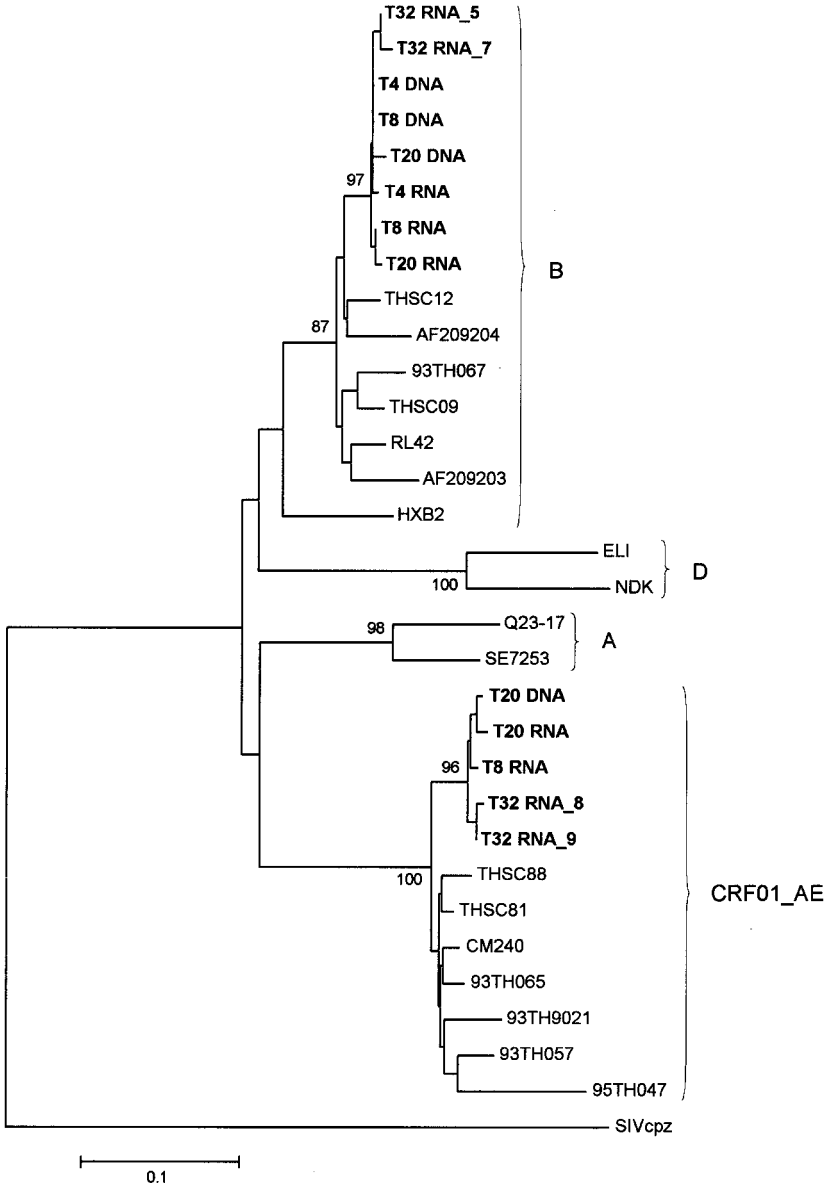


FIG. 2. Phylogenetic analysis of HIV-1 C2-V3 (283-bp) sequences from case 2 (bold) from T4, T8, T20, and T32 collection time points. Population sequences were obtained from the T4, T8, and T20 collection time points; subclone sequences were obtained from the T32 collection time point. The source of the sequence (PBMC-DNA lysate or cell-free RNA) is indicated after each collection time point where appropriate. Standard sequences representing HIV-1 subtypes were obtained from the HIV database at http://www.hiv-web.lanl.gov. THSC09 and THSC12 are representative subtype B sequences from the IDU cohort (35). THSC81 and THSC88 are representative CRF01_AE sequences from the IDU cohort (35). HIV-1 subtypes are indicated to the right of the trees, and bootstrap values are indicated to the left of the branch nodes. The trees are unrooted, and the branch lengths are proportional to the number of molecular changes between operational taxonomic units.

the CRF01_AE strain in cell-free virus at T8, and in all subsequent samples through T32 (Fig. 2). CRF01_AE-infected cells were first detected at T20, and in all subsequent samples through T32. Only CRF01_AE sequences from representative collection times were used in phylogenetic analysis (Fig. 2). Thus, in case 2, it appears that primary infection occurred with a subtype B strain. Superinfection with the CRF01_AE strain most likely occurred in the interval between T4 (19 January 1997) and T8 (25 June 1997).

Clinical parameters of HIV infection. We measured viral loads and $CD4^+$ and $CD8^+$ T-cell counts from FP (no CD4 or CD8 data were available for the FP sample) through T32 for both cases (Fig. 3). Throughout the follow-up of case 1, viral loads fluctuated at around 2 logs (range, 6,012 copies/ml at T20 to 72,719 copies/ml at T12). A similar 2-log fluctuation was seen in case 2 (range, 5,096 copies/ml at T24 to 80,183 copies/ml at T4). $CD4^+$ T-cell counts in case 1 remained below 500 cells/μl in seven of eight time points, while in case 2 the overall counts were higher and dropped below 500 only once, at T4. $CD8^+$ T-cell counts were constant for case 1 during the entire follow-up period (range, 612 to 1,273 cells/μl) but were highly variable for case 2 between visits (range, 959 to 2,510 cells/μl).

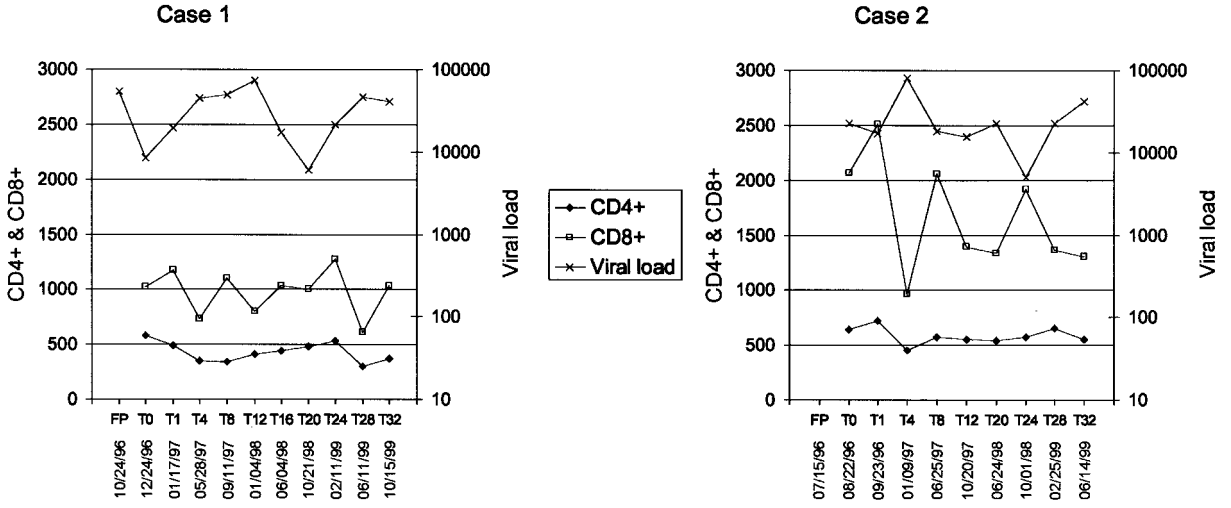


FIG. 3. Clinical parameters for disease progression for T0 through T32 for case 1 and case 2. Viral load is indicated on a log scale. For case 1, viral load was obtained for the FP time point also. For case 2, there was no detectable virus at FP. Viral load values are copies/milliliter; values for $CD4^+$ and $CD8^+$ T cells are per cubic millimeter.

In addition, case 2 had an overall decline in his $CD8^+$ T-cell counts, dropping from 2,510 to 1,302 cells/μl from T0 through T32.

V3 peptide-specific antibody responses. For case 1, serum antibodies to the CRF01_AE peptide were detected in all time points of follow-up (optical density range, 0.4 to 3.0) (Fig. 4a). In contrast, the subtype B-specific serum antibody reactivity was not detected in case 1 until T8 and was present through T32. This lag in detection of the B-peptide reactivity was consistent with the delayed detection of the subtype B strain from infected cells and cell-free virus, which was first seen at T1 (Fig. 4b). In case 2, serum antibodies reactive to the subtype B-specific V3 peptide were detected from T0 through T32. In contrast, serum reactivity to HIV-1 CRF01_AE peptide was

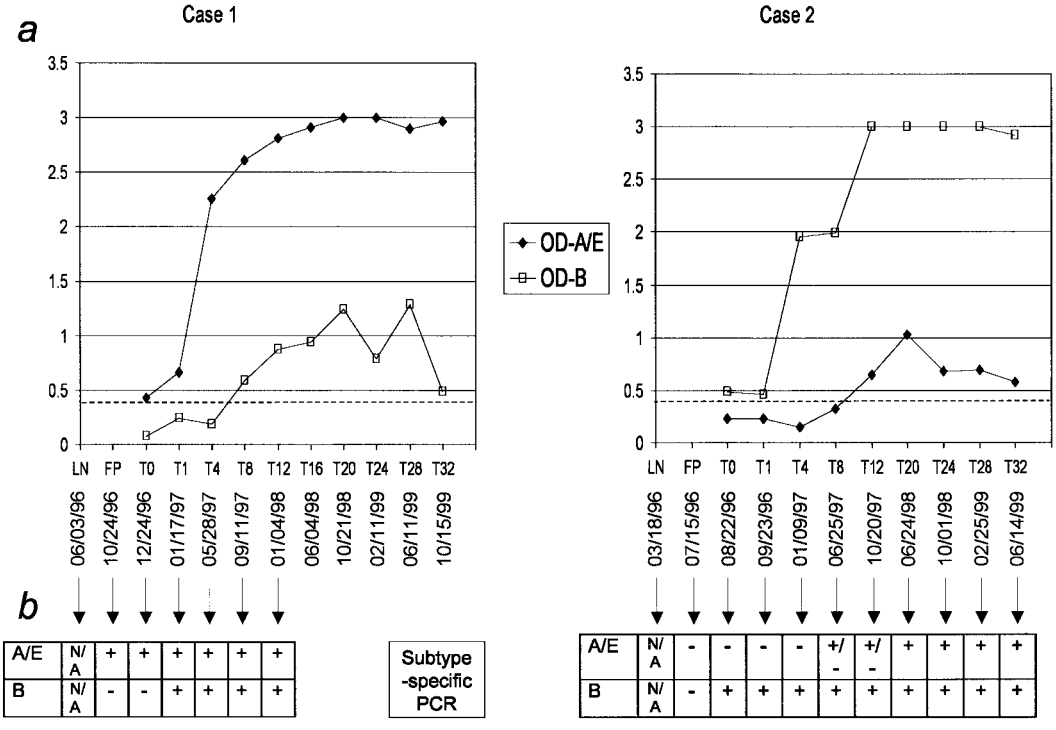
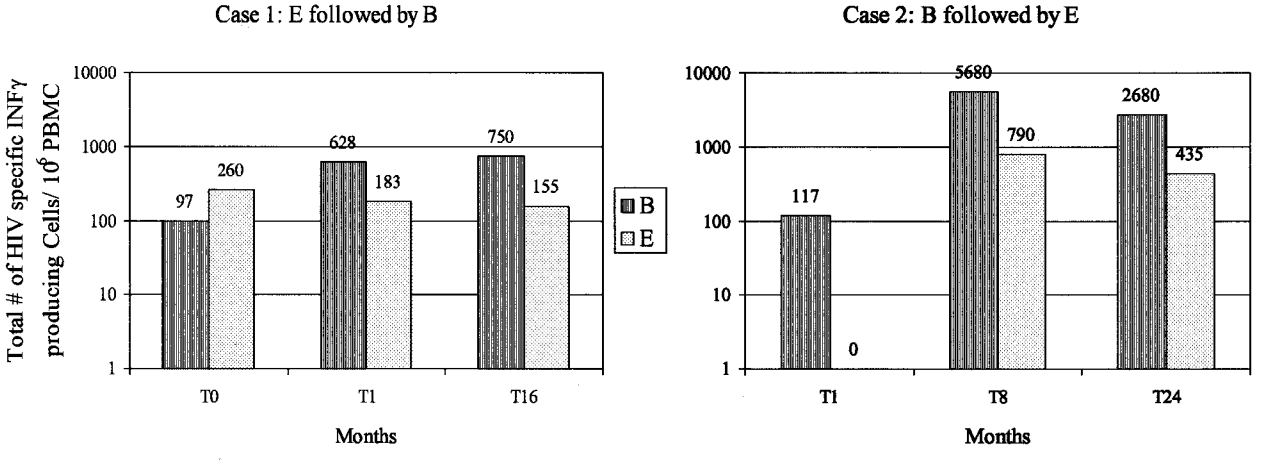


FIG. 4. EIA and PCR amplification for cases 1 and 2. (a) Absorbance was measured at 450 nm; a cutoff value of 0.3 (indicated by a dashed line) was used. (b) Subtype-specific amplification using CRF01_AE and subtype B-specific primers for case 1 (time points FP through T12) and for case 2 (time points FP through T32), respectively. +, positive amplification from both RNA and DNA; ±, positive amplification from RNA and negative amplification for DNA; −, negative amplification from both RNA and DNA; N/A, sample not available.



	T0				T1				T16			
	B		E		B		E		B		E	
	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)
Env	0	0	7	3	67.5	11	60	33	85	11	55	35
Gag	97	100	134	51	190	30	70	38	120	16	90	58
Nef	0	0	0	0	330	53	2.5	1	30	4	0	0
Pol	0	0	119	46	40	6	50	27	515	69	10	6

	T1				T8				T24			
	B		E		B		E		B		E	
	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)	SFU/10^6	(%)
Env	39	33	0	0	3240	57	460	58	2400	90	270	62
Gag	22	19	0	0	195	3	110	14	15	1	150	34
Nef	0	0	0	0	615	11	25	3	50	2	0	0
Pol	56	47	0	0	1630	29	195	25	215	8	15	3

FIG. 5. T-cell responses to recombinant vaccinia virus constructs expressing HIV-1 CRF01_AE and subtype B proteins. Counts of IFN-γ-producing T cells expressed as SFU/10^6 cells are indicated on top of each bar and on the vertical axis. Collection time points sampled are indicated on the horizontal axis. The tables below each bar graph show the CRF01_AE and subtype B T-cell responses for individual gene regions.

observed first at T8 and then at all follow-up times through T32 (Fig. 4a). This pattern was completely consistent with the initial detection of CFR01_AE-infected cells and cell-free virus at T8 (Fig. 4b).

HIV-specific cell-mediated immune responses. (i) Case 1. PBMC from T0, T1, and T16 were used to evaluate T-cell responses to vaccinia virus vectors expressing HIV-1 CRF01_AE and subtype B proteins, using IFN-γ ELISPOT. T-cell responses to vaccinia virus vectors expressing Gag and Pol of CRF01_AE were observed at T0 (260 spot-forming units [SFU]/10^6 PBMC) (Fig. 5). In addition, a low-level response (97 SFU/10^6 PBMC) to the vaccinia virus vectors expressing the subtype B Gag was also seen at T0, despite the absence of both molecular detection and a subtype B-specific V3 peptide antibody response at this time point (Fig. 4a and b). At T1, more than half a log increase in the number of T cells reacting to subtype B proteins (628 SFU/10^6 PBMC) was detected; however, with an increase in the level of response to B, the level of responses to CRF01_AE decreased slightly, resulting in a 50% reduction in the frequency of T cells specific to the CRF01_AE Gag and Pol. Additionally, a positive response to CRF01_AE Env occurred. Generally, the hierarchy of responses to CRF01_AE (Env, Gag, Nef, and Pol) from T1 to T16 did not change. However, a switch in the hierarchy of T-cell responses to subtype B proteins was seen: at T1 the response to Nef dominated, whereas the response to Pol became dominant at T16. Overall, the total number of T cells reactive to CRF01_AE dropped after the subtype B strain was acquired, from 260 to 155 SFU/10^6 PBMC. In contrast, the total number of T cells reactive to subtype B increased from 97 to 750 SFU/10^6 PBMC (Fig. 5).

(ii) Case 2. PBMC from T1 (only HIV-1 subtype B detected by subtype-specific PCR) and from T8 and T24 (both CRF01_AE and subtype B detected by subtype-specific PCR) were used to test T-cell responses. A low-level T-cell response to subtype B viral proteins was observed at T1 (117 SFU/10^6 PBMC), and T-cell responses to CRF01_AE-expressing vectors were not detected (Fig. 5). A broad T-cell response to all four CRF01_AE proteins was detected (790 SFU/10^6 PBMC) at T8, at which time the superinfection by CRF01_AE virus was first detected by molecular methods. In addition, a stronger, broader T-cell response to subtype B proteins was detected with a 1-log increase from the level seen at T1. Although from T8 to T24 there was no change in the hierarchy of subtype B and CRF01_AE responses toward all proteins within each subtype, a 50% decrease in the overall T-cell response to both B (5,680 to 2,680 SFU/10^6 PBMC) and E (790 to 435 SFU/10^6 PBMC) was observed (Fig 5). Unlike the T-cell responses in case 1, those in case 2 were skewed toward Env and the subtype B Env responses remained elevated (3,240 and 4,200 SFU/10^6 PBMC).

DISCUSSION

This is the first demonstration of potential HIV-1 superinfection in humans. Superinfection was observed in two IDUs, each of whom displayed strong humoral and cell-mediated immune responses to the primary virus. In each case, superin-

fecting heterologous viruses were detected more than 3 and 11 months after their respective primary infections. The vulnerability of the two individuals to superinfection, despite ostensibly intact immune systems and primary-virus-directed immune responses, raises concerns about the ability of current HIV-1 vaccines to protect against heterologous infection.

Our methods cannot completely exclude the possibility that the transmission of both subtypes occurred simultaneously or very close in time to one another during the seronegative window period following primary infection. We may not have detected the superinfecting strains, in each case, if they were present at levels below the PCR and antibody detection assay limits used in this study. To overcome any sampling bias during amplification, which could occur if the two subtypes were present in greatly disproportionate levels, subtype-specific *env* primers which were highly specific and sensitive were used to confirm each case of sequential infection. In addition, the patterns of V3 antibody and T-cell responses in both cases supported the molecular observations of superinfection. The superinfecting strains may have escaped detection until later in infection if they were compartmentalized or sequestered within specific tissues such as lymph nodes. Subtype-specific compartmentalization of HIV-1 has not been reported, and it is unlikely that compartmentalization would serve as an explanation for the inability to detect the superinfecting subtype in each case.

Several steps were taken both at the sample collection sites in Bangkok and during subsequent laboratory procedures to avoid sample mix-up. The strong clustering of sequences belonging to either CRF01_AE and subtype B, from different time points from case 1 and case 2 (Fig. 1b and 2), clearly demonstrated that sample mix-up did not occur. In addition, phylogenetic analysis showed that the superinfecting subtype B strain sequence in case 1 was distinct from the other 27 subtype B strains (data not shown) which had been previously identified in the cohort study (35), further ruling out sample mix-up. Similarly, the sequence of the superinfecting CRF01_AE strain in case 2 did not cluster strongly, as one would have observed if sequences were from the same individual, with any of the previously characterized 103 CRF01_AE sequences (35) from this IDU cohort study (data not shown). Therefore, it is very unlikely that the superinfecting strains in both cases were the result of sample mix-up.

A major issue hindering the development of preventive vaccines for HIV is a lack of understanding of the quantity and quality of immunity required to protect against viral challenge. Evaluating HIV-1 intersubtype superinfection offers a unique approach to examining the correlates of immune protection. A more detailed analysis of the humoral and cellular responses in these two cases could provide clues as to why the second infections occurred. From the present data, we can begin to elucidate some aspects of the humoral and cell-mediated responses that may have rendered both individuals susceptible to superinfection

Although we evaluated humoral responses only to the immunodominant V3 region of Env and currently do not have data on responses to other immunodominant proteins, the antibody data in both cases are consistent with the PCR data and support the notion of superinfection. Low-level, monoreactive subtype-specific V3 serology at early times following primary subtype infection in both cases may indicate an inability in these individuals to prevent superinfection with a heterologous strain through antibody-mediated mechanisms. In support of this hypothesis is the recent finding that in CRF01_AE-infected individuals, cross-neutralizing antibodies against subtype B were significantly higher in those who displayed dual (CRF01_AE/B) V3 serotypes (29). Thus, the lack of cross-neutralizing antibodies may have predisposed these two cases to superinfection.

For case 1, in contrast to the observed monospecific antibody response, the Gag-specific subtype B T-cell response at T0 could indicate that cross-reactivity to subtype B was induced by the primary CRF01_AE infection. If this is the case, then it is possible that superinfection occurred because this cross-reactivity was insufficiently broad or strong. In case 2, although broad T-cell responses to the homologous subtype B proteins (Env, Gag, and Pol) were detected at T1, cross-reactive T-cell responses to the CRF01_AE proteins were not seen. In this case, superinfection may have occurred because of the absence of cross-reactive T cells. However, in both cases, as T-cell responses to other proteins such as Tat, Vif, or Vpu were not analyzed, cross-reactivity may have been underestimated. Because of the sample collection schedule used in this cohort study, in evaluating T-cell responses at T0 and T1 we may have missed the peaks of the T-cell response which would have occurred soon after infection, and the patterns seen may reflect those of T cells in the posteffector or memory phase of the immune response (1). Additional studies of these and other cases could clarify the kind and extent of immune responses needed to prevent superinfection. Although studies from animal models (11, 12, 25, 33, 39) and our present data only partly define the correlates of protection, recent chimp superinfection studies provide promise that antigenic boosting may prevent superinfection and indicate an important role for cytotoxic T lymphocytes. (4).

While sterilizing immunity against HIV infection is an ultimate goal of HIV vaccine studies, recent data suggest that a more realistic goal may be to reduce virus loads to low or undetectable levels in vaccinees who become infected with HIV, as has been observed in several recent nonhuman primate studies (2, 5). The two cases of superinfection in our study also provide lessons relevant to this goal for HIV vaccine development. For example, if the virus load of the superinfecting strain was controlled by the immune responses to the primary virus, this would suggest that a vaccine could reduce the virus load and delay progression to AIDS. The clinical progressions (as monitored by virus loads and $CD4^+$ and $CD8^+$ T-cell counts) observed for case 1 and case 2 do not appear to be different from those of seroconverters in the BMA IDU study who were infected with only one subtype virus (data not shown), suggesting that the superinfection may not be deleterious in these individuals. It is interesting that in both case 1 and case 2, the optical density levels (reflecting subtype-specific antibody) for the second or reinfecting subtype rose above cutoff (optical density = 0.3) levels subsequent to PCR detection of the second subtype; however, the levels never reached the levels of the primary subtype. Moreover, in case 2, T-cell responses to the second virus were lower than those to the first virus. These data support the possibility that the first infection may have controlled the second infection. This lends

hope that vaccine-raised immune responses will control viremia following challenge, as was seen in recent macaque AIDS vaccine studies (2, 6). However, the virus can mutate and escape the vaccine-boosted immune system's attempts to control viremia, as was recently demonstrated in one of the nonhuman primate studies (6). HIV-1 superinfections in humans demonstrate that heterologous viruses can break through the immune response elicited by a natural HIV-1 infection. Continued follow-up of cases 1 and 2 and other such cases is needed to evaluate the emergence of recombinant viruses which may have altered phenotypic properties and to monitor the clinical progression of these individuals. Further studies of these two cases are being conducted to determine the relative proportions of each subtype and evaluate the changes in proportion over time.

While our data, showing that HIV superinfection is possible in humans, may raise a new challenge for HIV vaccine design, several caveats must be considered before translating directly from these superinfection studies to vaccines. First, the superinfections documented here occurred in IDUs, who most likely had direct intravenous inoculations of high viral doses, unlike most HIV infections worldwide which occur in persons who generally have a lower-dose mucosal challenge. Second, the nature of the immune response to primary HIV-1 infection differs from that of the immune response to a vaccine; i.e., during primary infection, although a broad immune response is generated, the loss of specific subsets of CD4 T cells creates a weakened immune system that may permit establishment of superinfection. Therefore, a vaccine-raised immune response may not have the same deleterious effects.

Given the evidence that superinfection with a genetically distinct strain is possible, additional cases of multiple subtype infection will likely be detected in this IDU cohort and perhaps in other cohorts as well. These two examples of superinfection have important implications for HIV-1-infected individuals who may be exposing themselves to superinfections with drug-resistant viruses. These two cases also help explain the high rates of unique recombinant HIV-1 genomes in regions with multiple subtypes. An important question for vaccine development is to determine to what extent differences between subtypes prevent induction of protective immune responses. Based on currently available data, there is no clear answer to this question. Our data could be interpreted to suggest that in some cases cross-recognition between HIV-1 CRF01_AE and B may not be adequate to prevent infection. Therefore, it may become necessary to match or tailor vaccines with the variants prevalent in a region. Alternatively, it may be more practical to design vaccines with protective epitopes that are shared by globally circulating HIV-1 variants.

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