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Longitudinal Studies of Viral Sequence, Viral Phenotype, and Immunologic Parameters of Human Immunodeficiency Virus Type 1 Infection in Perinatally Infected Twins with Discordant Disease Courses

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Perinatal human immunodeficiency virus type 1 (HIV-1) infections cause a broad spectrum of clinical disease and are variable in both the age of the patient at onset of serious disease and the progression of the clinical course. Heterozygotic perinatally infected twins with a marked difference in their clinical courses were monitored during the first 2 years of life. Twin B, the second-born twin, developed AIDS by 6 months of age and died at 22 months of age, while twin A remained minimally symptomatic through the first 2 years. Sequential blood specimens were obtained from the twins in order to characterize the immunologic properties of the children and the phenotypes and genotypes of the HIV-1 isolates at various times. Twin A developed neutralizing antibodies and a high-level antibody-mediated cellular cytotoxicity (ADCC) response, while twin B had no neutralizing antibody and a much lower ADCC response. The virus isolates obtained from the two children at various time points proliferated equally well in peripheral blood mononuclear cells, were non-syncytium inducing, and could not infect established T-cell lines. They differed in their ability to infect primary macrophages. In parallel to the biological studies, the HIV-1 *tat* and part of the *env* gene sequences of the longitudinal isolates at four time points were determined. Sequences of virus from both twins at different time points were highly conserved; the viruses evolved at a similar rate until the last analyzed time point, at which there was a dramatic increase in sequence diversity for the sicker child, especially in the *tat* gene. Our results show that the viruses isolated at different times do not have significant changes in growth properties. The absence or low levels of neutralizing antibodies may correlate with disease progression in the twins.

Perinatal human immunodeficiency virus type 1 (HIV-1) infection presents a unique setting for investigating HIV-1 pathogenesis. The timing of infection can be defined within a specific period, and events can be characterized sequentially. The evolution of disease in perinatally infected children is characterized by two different patterns (2, 20, 40). In the first, infants develop severe disease, including AIDS, within the first few months of life and their course progresses rapidly. This pattern is usually associated with a concomitant steep decline in CD4 cell numbers within the first 12 to 24 months of age. The natural history of disease associated with the second presentation is more variable, but the course of disease is generally more indolent, with the onset of serious disease often delayed for several years, and the CD4 cell numbers decline more slowly. Some children in this group survive into early adolescence.

It is not clear what factors are responsible for this difference in the evolution of perinatally acquired HIV-1 infection. The timing of perinatal infection, in utero or at delivery, as determined by the initial detection of virus at delivery or later, has been proposed as a determinant of disease onset and progression (12). In a study from the Centers for Disease Control and Prevention, infants with positive virus cultures or positive PCR at delivery had a greater risk for early and severe disease than infants in whom virus was not detected until after the first week of age (34). The severity of the maternal disease stage during pregnancy has also been directly associated with risk of early disease in infants (3, 40). In a study by investigators in France, infants born to mothers with AIDS had a substantially greater risk of having AIDS by 1 year of age than infants born to asymptomatic mothers (2). Whether this risk is related to virus burden, virus phenotype, or other factors has not been determined.

In adults, the appearance of virus with the ability to form syncytia or replicate to high titers has been associated with disease progression (9, 39). The role of viral phenotype in the evolution of disease in children has not been described. Other viral factors, such as genetic variation, could also be important in disease progression (25). Studies evaluating the genetic diversity of virus in perinatally infected infants have found a homogeneous sequence population in infants at birth (30, 42); others have suggested that multiple maternal genotypes are present in infected children (26). Very little, however, is known about the correlation between genetic variability and disease progression over time in adults or children.

Multiple factors are likely to alter the course of disease in children. The occurrence of divergent courses of disease in perinatally infected children provides the opportunity for identifying viral or immunologic factors that influence the natural history of perinatally acquired HIV-1 infection and disease. Perinatally infected heterozygotic twins with divergent clinical courses were monitored until almost 24 months of age. Sequential specimens from the twins were studied retrospectively to characterize the immune response and viral phenotype and

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genotype for each twin over time. Our results suggest that the absence of neutralizing antibodies can distinguish infants with differing disease courses.

MATERIALS AND METHODS

Cell cultures. Healthy-donor peripheral blood mononuclear cells (PBMC) were obtained from leukopacs (American Red Cross). The cells were purified on Lymphocyte Separation Medium (Organon Teknika, Durham, N.C.) and propagated in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 5 μg of phytohemagglutinin (Difco) per ml for 24 h before coculture with patient PBMC or infection with culture supernatant containing HIV-1. Primary monocytes were obtained from gradient-purified PBMC by the plastic adherent technique (7). Adherent cells were cultured for 7 to 10 days in RPMI 1640 medium containing 10% FBS and 10 ng of granulocyte-macrophage colony-stimulating factor (GIBCO) per ml to allow differentiation into macrophages.

Virus culture and phenotype and tropism determination. HIV-1 was isolated by standard coculture procedures (18). In brief, patient PBMC were cocultivated with an equal number of phytohemagglutinin-stimulated PBMC from an HIV-1-seronegative donor (final concentration, 2×10^6 PBMC per ml). Half of the culture medium was replaced with fresh medium twice a week, and equal numbers of fresh uninfected phytohemagglutinin-stimulated PBMC were added to the culture once a week. Virus production was monitored by measuring HIV-1 p24 antigen levels with a commercial enzyme-linked immunoassay kit (Coulter Corporation, Hialeah, Fla.). Virus stocks were prepared when p24 antigen exceeded 10 ng/ml, and titers were determined by limiting dilution to obtain the 50% tissue culture infectious dose ($TCID_{50}$) (see below).

Syncytium-inducing (SI) or non-syncytium-inducing (NSI) phenotype was determined by infecting MT2 cells in duplicate microtiter wells in a 96-well flat-bottomed plastic tissue culture plate with 50 μl of fresh virus stock (21). Virus was scored as SI if three to five syncytia were observed per well within a 14-day period and as NSI if syncytia failed to form in that time.

To determine viral tropism for various host cell types, human PBMC, primary macrophages, and T-cell lines CEM and H9 were infected with the virus stock by a standard method (27). Briefly, cells were treated with 2 μg of Polybrene per ml (1 h at 37°C) and then incubated with virus stock (10 to 15 ng of HIV-1 p24 antigen per 5×10^5 host cells; incubation for 2 h at 37°C), washed, and suspended in fresh culture medium. Supernatants were removed at various times postinfection and assayed for HIV-1 p24 antigen.

Virus titration and $TCID_{50}$ calculation. Stocks of virus obtained from the children at the different time points were thawed and serially diluted fivefold, starting with a dilution of 1:5. Two hundred microliters of each virus dilution was incubated in duplicate with 0.3 ml of pooled PBMC containing 10^6 cells in eight-strip cluster tubes (Costar, Cambridge, Mass.) for 1 h at 37°C in a 5% CO_2 incubator. The cells were washed twice, resuspended to 0.2 ml with complete medium, and transferred to 48-well plates containing 0.8 ml of medium per well.

Four days after infection, 0.5 ml of culture supernatants from each well was replaced with 0.5 ml of supernatant fresh medium. At days 7 and 10 postinfection, 0.5 ml of supernatant in each well was replaced with 0.5 ml of PBMC suspension containing 10^6 cells. Virus production was estimated at day 14 postinfection by measuring the HIV-1 p24 antigen level. One $TCID_{50}$ unit was defined as the virus dilution required to infect half of the replicate cultures.

Neutralization of autologous virus. Plasma specimens were heat inactivated at 56°C for 30 min and serially diluted fourfold in complete medium, starting with a 1:5 dilution. Fifty microliters of each plasma dilution was incubated in duplicate with 0.2 ml of 100, 50, 25, or 12.5 $TCID_{50}$ units of autologous virus cultured from the same time point, at 37°C for 1 h in 5% CO_2. Two hundred fifty microliters of pooled PBMC containing 10^6 cells was added, and the mixture was incubated overnight. The cells were then washed twice, resuspended in 0.2 ml of complete medium, and transferred to a 48-well tissue culture plate containing 0.8 ml of medium per well. Four and seven days later, 0.5 ml of culture supernatants was removed for p24 antigen determination. Medium was replaced on day 4, and on day 7 the culture was terminated. Samples with p24 levels above the range of the assay were diluted and reassayed. For each virus input and plasma dilution, the percent inhibition was calculated as follows: 100 − (p24 antigen production in tubes containing serum/p24 antigen production in tubes with no serum) × 100.

HIV-1 ADCC assay. Antibody-dependent cellular cytotoxicity (ADCC) antibodies specific for HIV-1 Env protein were measured by a standard chromium release assay. D-2 cells, chemically transformed mouse fibroblasts (a gift from Thomas Leist, Cornell Medical Center), infected with a vaccinia virus recombinant, vPE8 (AIDS Reference Reagent Repository), were used as target cells. D-2 cells infected with vaccinia virus and uninfected D-2 cells were also used in each assay as controls. To prepare the target cells, D-2 cells were grown to semiconfluency in RPMI 1640 medium with glutamine, 10% FBS, and 0.1% gentamicin for 24 h. The cells were trypsinized and washed, and 2×10^6 cells were labelled with 200 μCi of ^{51}Cr for 1 h at 37°C. After being labelled, the cells were infected with the recombinant virus or control vaccinia virus at a multiplicity of infection of 10 PFU per cell in a volume of 1.0 ml of medium. A third population of uninfected control cells was adjusted to a total volume of 1.0 ml and thereafter treated similarly to the infected cells. After incubation at 37°C (5% CO_2) for 2.5 h, the cells were washed three times in RPMI 1640 medium with 10% FBS and resuspended to a final concentration of 10^4 cells per 50 μl. For the assay, 10^4 cells were added per well. PBMC from a healthy uninfected donor previously determined to have a high level of ADCC activity were separated with Lymphocyte Separation Medium and washed three times, and the cell concentration was adjusted to provide effector-to-target cell ratios as indicated below (see Fig. 1). Three different effector-to-target cell ratios were used in each assay to verify the specificity of the lytic activity observed.

Sera were heat inactivated, and 50 μl of serum was added to each well. Target cells were incubated with sera at 4°C for 45 min. A single concentration of serum (1:200, final dilution) was tested in each assay. In addition to the test sera, each assay also included vaccinia virus-positive sera and vaccinia virus-negative and HIV-1-negative sera as controls. For the assay, control and test samples were tested in duplicate in 96-well U-bottom microtiter plates. Spontaneous release and maximum release were each tested in six wells, and a mean value was calculated for each. Serum was first added to the wells, and then 50 μl of target cells and 100 μl containing the effector cells were added. The plates were incubated for 6 h at 37°C in 5% CO_2 and centrifuged at $400 \times g$ for 10 min. Fifty microliters of supernatant from each well was transferred to a counting tube for measurement of ^{51}Cr release. For each serum sample tested, lysis was calculated as follows: [(average cpm of sample − average cpm of spontaneous release)/(average cpm of maximum release − average cpm of spontaneous release)] × 100.

To determine HIV-1-specific cytotoxicity, percent lysis of the vaccinia virus-seropositive or vaccinia virus- and HIV-1-seronegative sera (whichever was greater) was subtracted from percent lysis of the test sera.

DNA preparation, PCR analyses, and cloning. High-molecular-weight DNA was obtained from infected cells by a standard proteinase K digestion method (1). Briefly, 1×10^7 to 3×10^7 infected cells were resuspended in 0.3 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per ml) and incubated at 50°C overnight. The digested samples were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) overnight, and the DNA concentration was quantified by UV absorbance. The HIV copy number of each DNA preparation from different time points was determined by semiquantitative PCR by methods described previously (43). DNA samples containing about 500 HIV-1 target molecules per sample were used for PCR.

All the primers for PCR were designed by using the HIV-1 conserved sequences published in the Human Retrovirus and AIDS Sequence Data Base (31). The positions of each primer correspond to the positions of HIV-1 HXB2 viral sequence. Primers for amplification and cloning of the viral envelope V3 loop and CD4 binding region are as follows: HENV-1, 5′-GTATGAATTCAACTGCTGTTAAATGGCAGT-3′ (positions 6986 to 7015; an *Eco*RI site is shown underlined); and HENV-2, 5′-TATAGAATTCACTTCTCCAATTGTCCCTCAT-3′ (positions 7676 to 7646; an *Eco*RI site is underlined). HENV-1, HENV-2, and HENV-5 (5′-GTTTTATTCTGCATGGGAGTGTGAT-3′; positions 7487 to 7463) were used as primers for performing DNA sequence analyses. TAT-F (5′-TTTCAGAATTGGATGCCGACATA-3′; positions 5771 to 5793) and TAT-R (5′-GGACCACACAACTATTGCTATTATT-3′; positions 6132 to 6108) were used to amplify and clone the viral *tat* gene exon 1.

PCR mixtures consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 20 pmol of each primer, 1 μg of high-molecular-weight DNA, 0.25 mM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase. The reaction was carried out in a total volume of 100 μl. The first cycle was 94°C for 3 min, 72°C for 2 min, and 50°C for 1 min 30 s and was followed by 35 cycles of 94°C for 45 s, 72°C for 2 min, and 55°C for 1 min. The last cycle was 94°C for 45 s and 72°C for 10 min. The PCR products were separated on a 1% agarose gel and purified by electroelution. The purified DNA fragments were ligated to the pGEM T-vector according to the procedures recommended by the manufacturer (Promega, Madison, Wis.).

DNA sequencing and analysis. Sequence data were obtained by using a commercial sequencing kit (U.S. Biochemicals, Columbus, Ohio) based on the dideoxynucleotide termination method (35). Sequence analyses were carried out with the Genetics Computer Group, Inc. (Madison, Wis.), sequence analysis package. DNA sequences were also aligned manually. Nucleotide gaps were assigned after amino acid conversion to maintain translation integrity. Nucleotide gaps and insertions were commonly found in the V4 region even in different sequences from the same individual. Nucleotide distances were determined by using the DNASTAR program (DNASTAR, Madison, Wis.). Alignment gaps were not counted as differences.

Phylogenetic analyses were performed by using the program PHYLIP, version 3.5c (13). Five hundred bootstrap sequence sets were generated, parsimony analyses were performed with universal branch swapping, and a consensus tree was constructed. A time scale was added to the trees by using the dates of sample collection (19).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *env* sequences are U47562 to U47588, and the numbers for the *tat* sequences are U47589 to U47613.

TABLE 1. Clinical histories of the twins[a]

Date (mo/yr)	Age (mo)	Finding(s) for twin[b]:		Absolute CD4 T-cell no./mm^3	
		A	B	Twin A	Twin B
04/92[c,d]	1.5	Normal exam	Normal exam	1,882	699
06/92[c,d]	3	LAD	Hepatomegaly, LAD	ND[e]	ND
10/92	6	HSM	Hepatomegaly, LAD, esophagitis, AIDS	1,774	94
01/93[c,d]	10	LAD, HSM	LAD, hepatomegaly, developmental delay	ND	ND
04/93[c,d]	13.5				
08/93[d]	16	Recurrent otitis media, LAD, HSM	Pneumonia and sepsis, hepatomegaly, developmental delay	1,347	64
12/93	20	Not seen	Pneumonia and sepsis, esophagitis, herpes simplex stomatitis, developmental delay	ND	ND
02/94	22	Chronic otitis media, LIP, LAD, HSM	Acute respiratory failure, death	ND	ND
05/94[d]	25	Chronic otitis media, LAD, HSM, LIP		1,556	

[a] Both twins are female.
[b] LAD, lymphoadenopathy; HSM, hepatosplenomegaly; LIP, lymphoid interstitial pneumonitis.
[c] HIV-1 genetic analyses were performed.
[d] HIV-1 cell tropism analyses were performed.
[e] ND, not determined.

RESULTS

Clinical disease course. Twins A and B were born to a 29-year-old multigravida mother who received no prenatal care and whose own infection was not diagnosed until after the birth of the twins. The mother had no history of drug use or previous blood transfusions. The twins have several siblings, but none were HIV seropositive. The infants were delivered by cesarean section because of failure of labor to progress. Twin A, the healthier twin, was born first, weighing 3.41 kg, and twin B weighed 3.0 kg. Because of maternal endometritis and a positive urine antigen test for group B streptococcus in both infants, both received antibiotics for 10 days. Their nursery course was not remarkable otherwise.

The infants were first tested for HIV-1 at 6 weeks of age. Virus cultures from both infants were positive. The infants, however, were asymptomatic at this time, with normal physical examinations (Table 1). At 6 weeks (1.5 months), the CD4 count was already significantly lower for twin B (699/mm^3) than for twin A (1,882/mm^3). Twin B subsequently developed failure to thrive, esophagitis at 6 months of age, and encephalopathy. She had numerous infections and was hospitalized six times before her death at 22 months of age. In contrast, twin A had minimal clinical symptoms (lymphadenopathy, hepatomegaly, and chronic otitis media and radiographic evidence of lymphoid interstitial pneumonitis) during the first 24 months of life, and her CD4 counts remained >1,500/mm^3.

ADCC. Lysis of cells expressing HIV-1 gp120 by ADCC was measured for both twins, with serum samples obtained at 1.5, 10, 13.5, and 20 months of age (Fig. 1). At 1.5 months, the degree of lysis was very low for both infants (10% or less). At 10 months, the level of lysis was distinctly different between the twins and remained different in all sequential serum specimens tested. Sera from twin A, the healthier twin, had >40% lysis at 10 months, but lysis in twin B was still only about 10%. At 13.5 and 20 months, lysis of cells by serum from twin B was almost 20%, but this level was at least one-third less than that measured for twin A.

Neutralizing antibodies. Autologous neutralizing-antibody reactivity in the plasma of the twins was tested. For twin A, plasma samples were available at ages 1.5, 3, and 25 months. For twin B, autologous neutralization was tested for virus-plasma pairs at 1.5, 3, 10, and 13.5 months of age. As shown in Fig. 2, twin A had no neutralizing antibodies at 1.5 months, but by 3 months of age neutralization of the autologous isolate was already evident. The levels of neutralizing-antibody reactivity increased with time, and at 25 months of age, the level of neutralizing-antibody reactivity was higher than in the previous plasma samples. Twin B did not develop any measurable neutralizing antibodies in any of the sequential plasma samples tested, suggesting that the absence of neutralizing antibodies may correlate with disease progression.

Viral growth properties and host cell tropism. Studies were carried out to examine the growth properties of the different viral isolates from the twins at various times. Five isolates from each child were studied. For twin A, isolates taken at 1.5, 3, 10, 13.5, and 25 months were studied. For the sicker twin (B), isolates taken at 1.5, 3, 10, 13.5, and 16 months were studied. The viruses, isolated from minimally cultured PBMC, were further characterized for their growth characteristics. The viruses isolated from the two children at various times grew equally well in PBMC (Table 2). They were all NSI, and none of the isolates grew in CEM or H9 T-cell lines. Even the last available isolate obtained from twin B, at 16 months, prior to death, displayed no SI phenotype and could not grow in H9 cells. All the isolates from the five time points from the healthier twin A grew in macrophages but less well than positive-control macrophage-tropic HIV-1 isolate 128A. In contrast, for the sicker twin (B), viruses from the earlier time points grew very well in macrophages, similar to the positive control. However, their ability to grow in macrophages decreased with time, and by 16 months, the virus displayed only minimal macrophage tropism. These differences in tropism are unlikely to be due to differences in input virus titer, because the p24 titers and $TCID_{50}$ of the viruses were equalized before infection. In addition, subsequent passages of virus from each time point for both children were tested, and their macrophage tropism was confirmed (data not shown).

Molecular cloning and sequence analyses. In parallel with our studies of the biological properties of the HIV-1 isolates, the genetic sequences of the longitudinal viral isolates were also determined. The *env* gene from each isolate at four time points for each twin was cloned and sequenced; the region analyzed included the V3, V4, and CD4 binding domains (Fig. 3). In addition, the *tat* gene sequences of the longitudinal isolates were also determined in order to obtain an estimate of the rate of evolution of the virus in the absence of the strong

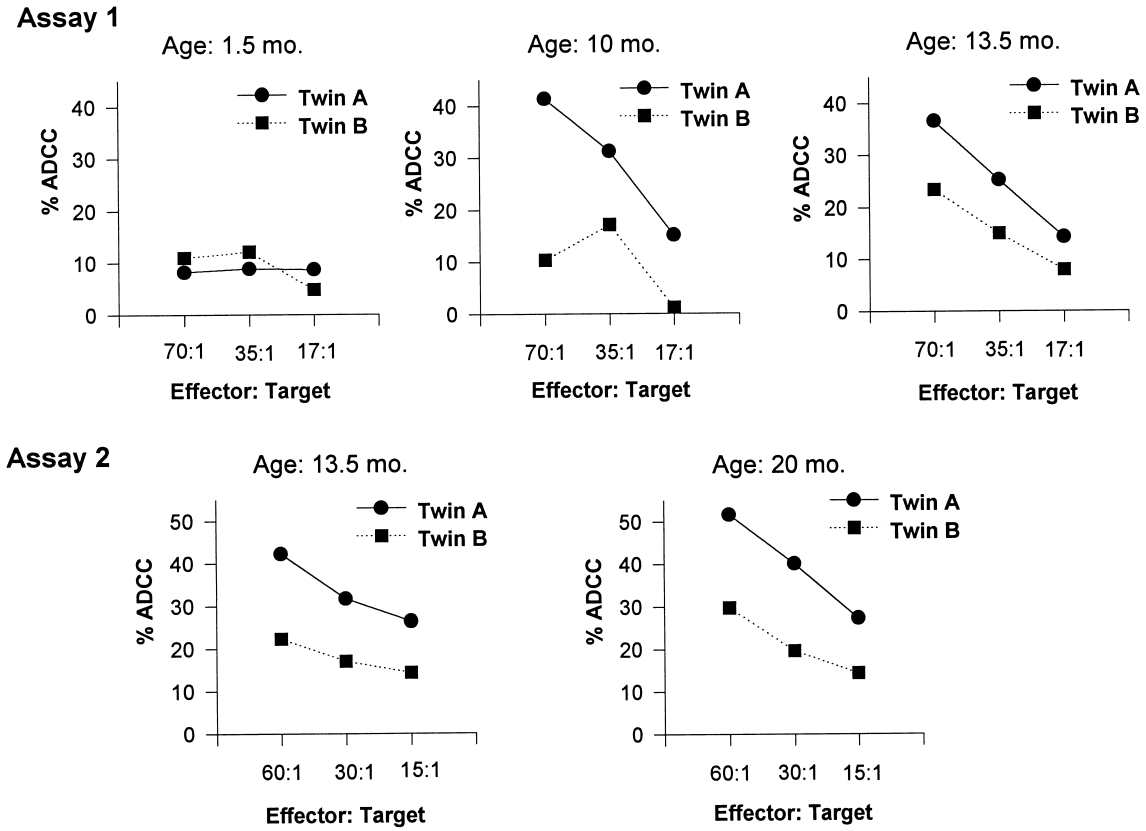

FIG. 1. Comparison of ADCC activities of the twins' serum samples obtained at various time points. Percent ADCC was calculated from the amount of chromium released after addition of the sera as described in the text. Different effector-to-target cell ratios were used in two separate assays to confirm the specificity of lysis. mo., months.

selection pressure reflected by changes in the *env* gene (Fig. 4). Five *env* clones and at least five *tat* clones from each isolate at each time point (a total of 40 *env* clones and 42 *tat* clones) were sequenced and aligned. Because of gaps and insertions, the nucleotide lengths of the *env* sequences varied from 526 to 570 bases. There were 27 different *env* forms among the 40 clones. One *env* sequence had a mutation which resulted in a frameshift. The *tat* sequences were uniform in length (219 bases). There were 21 different *tat* forms among 42 clones sequenced. The nucleotide sequences from different time points and from different twins were highly conserved. Phylogenetic analysis comparing the *env* V3, V4, and CD4 nucleotide sequences shows that even though the sequences from the twins are highly conserved, sequences from each twin seem to cluster more closely to each other and are distinct from the clade B consensus sequence (data not shown). The most diverse sequences of the different *env* genes from the twins still had about 97% identity. The intraindividual differences in nucleotide sequence between isolates from different time points are shown in Table 3. The *env* gene from each of the twins had approximately the same rate of diversification from the first to the last time points (1.9×10^{-2} nonsilent changes per nucleotide per year for twin B and 1.7×10^{-2} for twin A). The silent mutations from the first to last time points are 7×10^{-3} changes per nucleotide per year for twin B and 4×10^{-3} for twin A. In contrast, the *tat* gene for the sicker twin B had 6 times more nonsilent changes than twin A (1.9×10^{-2} changes per nucleotide per year versus 3.2×10^{-3}) and 4.5 times more silent changes than twin A (7.3×10^{-3} changes per nucleotide per year versus 1.6×10^{-3}), with most of the differences occurring at the last time point.

When the V3 sequences were aligned, all the isolates displayed sequences that were found in typical macrophage-tropic HIV-1 isolates. There are four different forms of V3 sequences among the sequenced clones. They are represented by clones A1-E1, A3-E1, A10-E1, and B13-E5 (Fig. 3 and 5). No significant differences in the V3 region were observed between isolates that displayed macrophage tropisms (twin B) and those that did not (twin A) (Fig. 3). Our sequences nevertheless are more similar to those of cloned HIV-1 strains that display macrophage tropism (SF128A, JRFL, BAL1, and SF162) than to those of strains that display T-cell tropism (SF2, IIIB, and NL43). Several amino acids (positions 13, 23, and 27) that were found to be conserved in many different macrophage-tropic strains were also found in our clones (Fig. 5) (8). It has been suggested that the overall charge of the V3 loop at pH 7 correlates with the ability of HIV-1 to induce syncytia (14). Strains with higher charges can induce syncytia, and those with lower charges cannot. Interestingly, the overall charge of the V3 loop of our clones from both twins is found to be around 3. These low net charges correlate well with the NSI phenotype of the isolates (Fig. 5).

To further determine the relationship between the HIV-1 clones from different time points, the sequences were also analyzed by neighbor-joining and parsimony analyses. An evolutionary tree was created from the parsimony analysis of these sequences (Fig. 6). This tree shows the heritage of progeny virus forms derived from earlier forms and the discontinuance

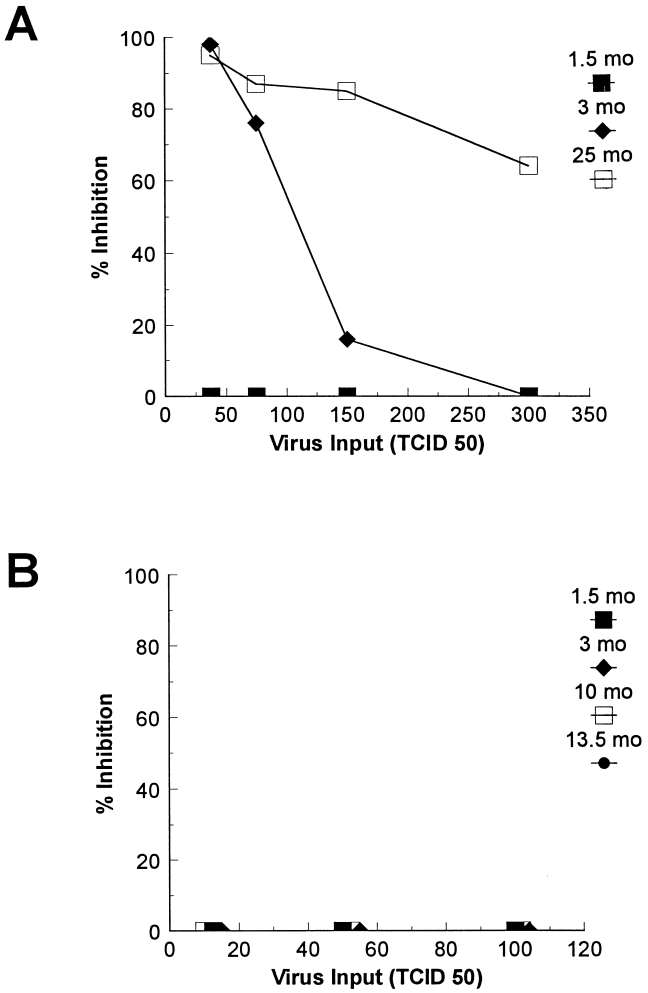

FIG. 2. Neutralization of the twins' viral isolates by their autologous sera (1/20 dilution) at various times. Percent inhibition of p24 antigen production is shown for different amounts of input virus as measured on day 7 after infection. (A) Twin A; (B) twin B.

of other branches. The divergence among viral forms in the tree branches is represented by their vertical spread. For the *env* gene sequences for twin A, the first time point began with two distinct viral forms. However, only one viral form was carried through to the last analyzed time point. Interestingly, for twin B, the sicker child, greater diversity of the viruses was observed at the fourth time point. Especially for the *tat* gene, there was a dramatic change between time points 3 and 4, and the viruses diversified and formed a distinct group. Separate phylogenetic analyses using the neighbor-joining method of linking maximum-likelihood distances confirmed these findings for the *env* and *tat* genes (data not shown).

DISCUSSION

In these perinatally infected twins, clinical courses that were distinctly different were noted within a few months after birth. The different patterns of disease that occurred in these infants were characteristic of the bimodal patterns of clinical disease that have been described previously for children with perinatal HIV-1 infection (2). One of the children, who developed an AIDS-defining disease by 6 months of age that was associated with a rapid decline in CD4 cell number, had a pattern of early-onset disease with rapid progression. In comparison, her twin was relatively healthy during the first 24 months of life, with CD4 cell counts that were normal for her age. Her course was typical of children having the second pattern of disease, which is characterized by a more indolent disease process and a comparatively longer survival.

Various factors have been proposed to explain differences in the rate of disease progression between perinatally infected children, including the rate of disease progression in the mother at the time of infection (2, 40), the ability of the infant to mount an immune response following infection (5), and biological properties of the infecting virus (11, 39). In addition, it has been shown that the timing of HIV-1 transmission from mother to infant (in utero, at birth, or postpartum) correlates with the rate of disease progression (12). Whatever the underlying cause of the difference, the twins in this study provided the opportunity to compare specific virologic and immunologic factors that may play a role in the differing patterns of disease that occur in children. Our longitudinal and parallel characterizations of humoral immunity and viral diversity and biologic properties suggest that both immunologic and viral factors may distinguish the two disease courses.

Since this was a retrospective study, not all relevant specimens were available. Specimens from the mother and infants at birth and from the twin with early disease just prior to death would have provided more-detailed information about the role of virus phenotypes and genetic diversity in evolution of the disease. In addition, the timing of perinatal transmission and the role of viral burden in the two infants at birth could not be examined, because birth specimens were not available. For most time points studied, only viral isolates from cells that had undergone minimal time in culture were available for study.

Our studies that compared the development of autologous neutralizing antibodies and ADCC found clear differences between the two infants. Using contemporaneous virus isolates and plasma specimens, we showed that neither infant had antibodies capable of neutralizing autologous virus at 6 weeks of age. By 3 months, however, the healthy twin had developed neutralizing antibodies, while these antibodies were not present in serum from the twin with early onset of disease at 3 months of age or in any subsequent sample studied. The lack of neutralizing antibodies at 6 weeks of age in both twins suggests that maternal neutralizing antibodies to the transmitted virus were absent at birth and therefore were absent in the

TABLE 2. Host cell tropism of various viral isolates from the twins

Isolate	P24 antigen production in the indicated host cells[a]			
	PBMC	Macrophages	CEM	H9
128A	740,000	1,582	—[b]	—
SF2	32,550	19	—	48,570
A1[c]	726,400	149	11	1
A3	817,500	160	21	0
A10	375,300	319	5	2
A13	489,500	278	2	0
A25	—	154	—	2
B1	731,500	1,764	4	0
B3	781,600	2,022	3	0
B10	528,700	3,521	9	0
B13	362,700	839	4	2
B16	—	142	—	0

[a] Given in picograms of p24 antigen per ml measured after 14 days in culture.
[b] —, not tested.
[c] A1, viral isolate from twin A at 1 month of age; other isolates are designated similarly.

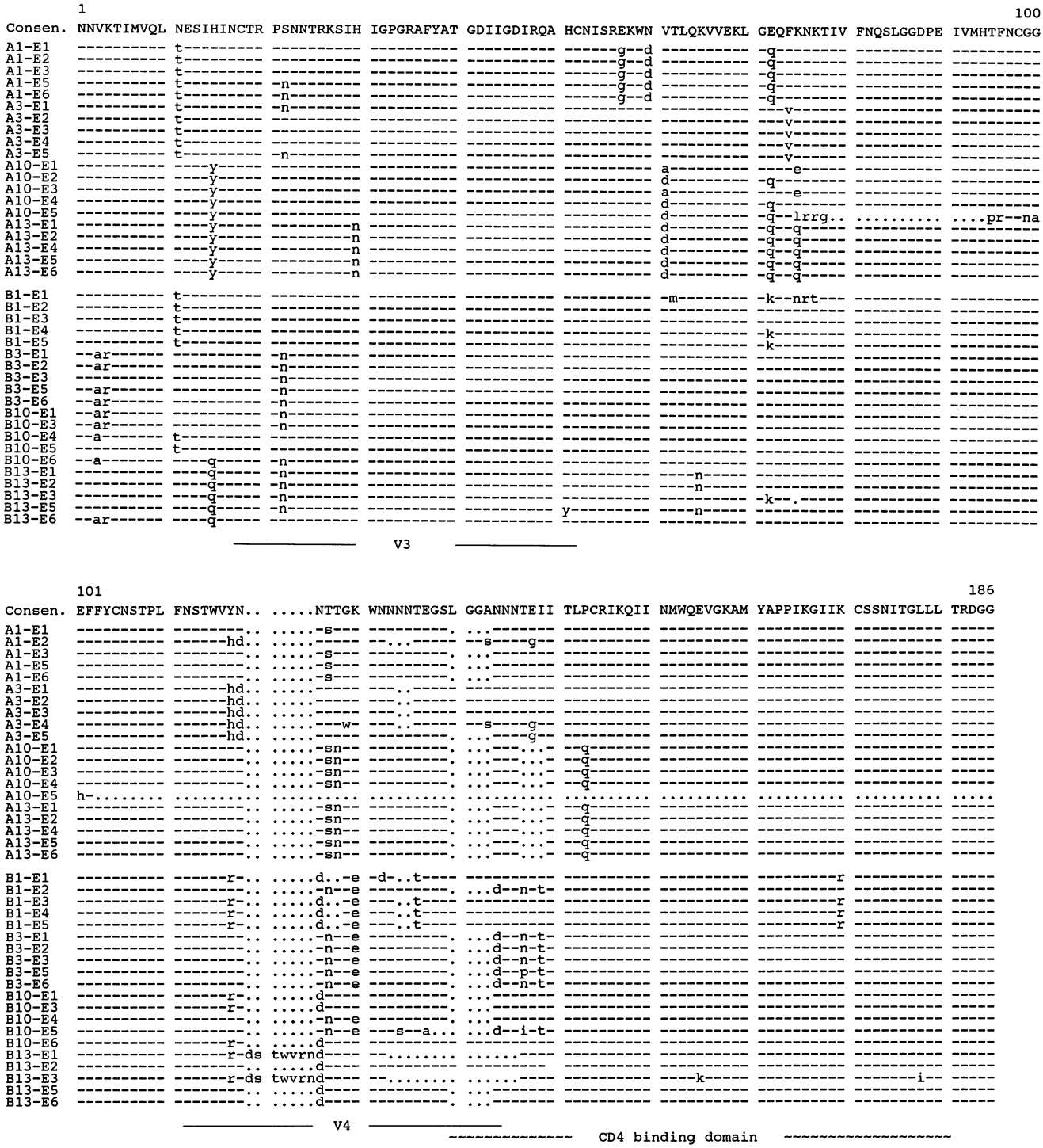

FIG. 3. Alignment of the envelope gene amino acid sequences from the V3 to CD4 binding domains of the various HIV-1 isolates from the twins. Five clones from each twin at each time point were sequenced. A1-E1, envelope clone 1 from twin A isolate at 1 month of age; A3-E1, envelope clone 1 from twin A isolate at 3 months of age; other clones are designated similarly. The consensus sequence (Consen.) of the V3 to CD4 binding domain region is listed at the top. Amino acids identical to the consensus sequences (−), those that are different (lowercase), and gaps (dots) are indicated.

mother. This is in agreement with recently published studies correlating the absence of maternal neutralizing antibodies and a higher risk of infection of the infant (23, 36). De novo production of antibodies by 3 months of age in the healthier twin is consistent with reports indicating that new antibodies to HIV-1 are detectable in some children between 3 and 6 months of age (17, 33). The presence of an autologous neutralizing antibody in infant specimens collected between 0 and 3 months has been investigated previously (23). In that study, no neutralizing antibodies were found in any of four children tested.

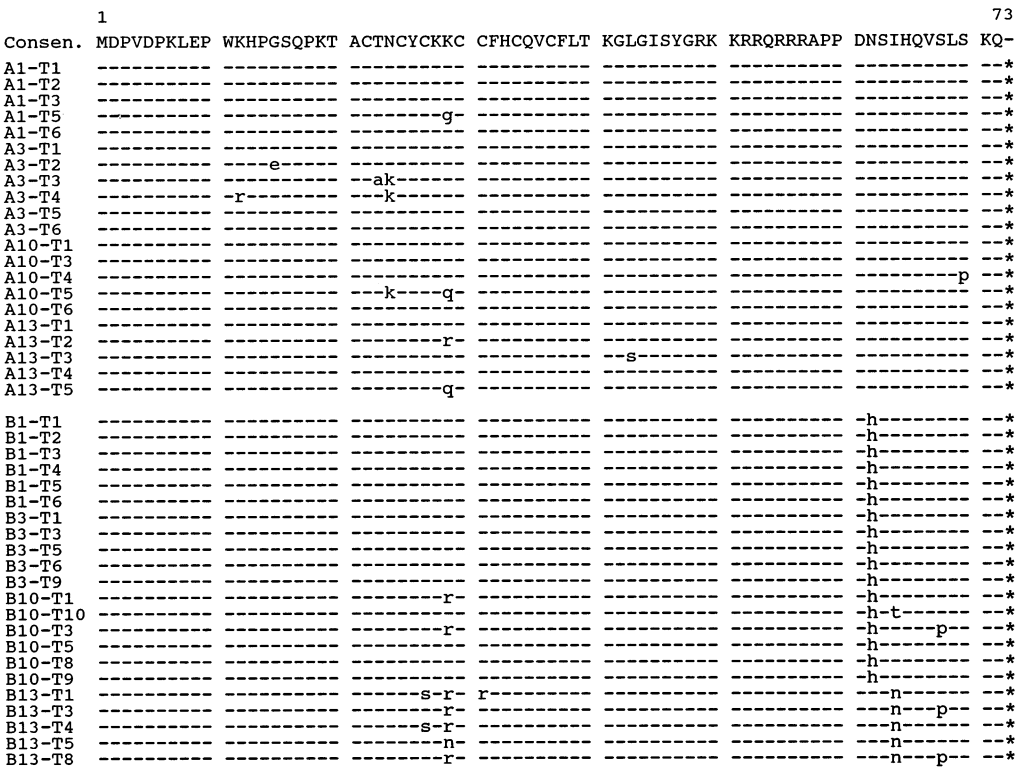

FIG. 4. Alignment of the *tat* gene product amino acid sequences from the various HIV-1 isolates from the twins. Five clones from each twin at each time point were sequenced. A1-T1, *tat* clone 1 from twin A isolate at 1 month; other clones are designated similarly. See the legend to Fig. 3 for details. Consen., consensus sequence; *, stop codon.

TABLE 3. Nucleotide distances of twin A and B HIV-1 isolates at different times

Gene segment and isolate[a]	Internal variation (%)[b]	% Changes[c]
V3-V4		
A1	0.64	
A3	0.53	2.13
A10	0.4	1.96
A13	0	2.09
B1	1.34	
B3	0.5	2.51
B10	1.51	2.22
B13	1.21	2.69
Tat		
A1	0.55	
A3	1.19	1.10
A10	0.55	0.55
A13	0.50	0.46
B1	0	
B3	0.18	0.09
B10	0.96	0.55
B13	1.74	2.65

[a] A1, viral isolate from twin A at 1 month of age; other isolates are designated similarly.

[b] Average percent nucleotide distance between clones from the same isolate at each time point.

[c] Percent nucleotide distance from sequence obtained at the first time point for the same twin.

Thus, this is the first report documenting the development over time of an autologous neutralizing-antibody response in children in correlation with disease progression. Even though no autologous neutralizing-antibody response was found in the sicker twin as determined by using contemporaneous plasma specimens, it is possible that a delayed response to the virus isolates occurred later. Because only a limited amount of plasma from either twin was available, this question could not be further explored. In addition, the lack of development of autologous neutralizing antibodies in the sicker twin is not a reflection of an absolute lack of de novo synthesis of antibodies, as indicated by the detection of ADCC in this twin by 10 months of age.

The ability to form syncytia has been shown to correlate with disease progression in adults (9, 39). Very little information is known about this factor in children. Recent studies have suggested that infants with viral isolates classified as rapid or high-level virus producers and having the SI phenotype are more likely to have rapid disease progression, while children with slow or low-level virus production and NSI viral isolates are likely to have a more indolent disease course (11, 38). With our progressor twin B, even at the last time point characterized, the virus was NSI, displayed a low level of cytopathology, and was unable to grow in T-cell lines; however, the last viral isolate from twin B (fifth time point) seemed to be losing its macrophage tropism. It cannot be ruled out that, prior to the death of twin B, her virus may have acquired T-cell tropism, SI, and a rapid- or high-level-producer phenotype.

Evolution of viral phenotypic properties can be attributed to changes in HIV-1 sequence (31). The variable domains of the gp120 *env* gene contain important determinants for viral host cell tropism, for cytopathology in culture, and for host immune

Sequence	Tropism	Syncytia	Charge
1 11 21 31 37			
SF2 ---------- --y-..---- --h---r--- ---k---	T	+	6.36
HTLV-IIIB ---------- k-r------- --v-i-k.-n m-----	T	+	9.12
NL43 ---------- --r------- --v-i-k.-n m-----	T	+	8.12
SF128A ---------- --n-..---- -i---a--- -------	M	-	4.12
JRFL ----s----- ----..---- ---------- -------	M	-	3.36
BAL1 ---------- ----..---- ---------- -------	M	-	3.36
SF162 ---------- --t-..---- ---a------ -------	M	-	3.12
A1-E1 ----s----- ----..---- ---a------ -------		-	3.36
A3-E1 ---------- ----..---- ---a------ -------		-	3.36
A13-E1 ----s----- --n-..---- ---a------ -------		-	3.12
B13-E5 ---------- ----..---- ---a------ -----y-		-	3.12
Consensus CTRPNNNTRK SIHIQRGPGR AFYTTGDIIG DIRQAHC			

FIG. 5. Alignment of the V3 sequences of the HIV-1 isolates from the twins. Amino acids identical to the consensus sequence (dashes), those that are different (lowercase), and gaps (points) are indicated. Amino acids that were suggested to play important roles in determining HIV-1 macrophage tropism (8) are shaded. HIV-1 strains SF2, HTLV-IIIB, and NL43 are T-cell tropic (T). HIV-1 strains SF128A, BAL1, JRFL, and SF162 are macrophage tropic (M). The overall charge of each V3 domain was calculated at pH 7 by using the ISOELECTRIC program in the Genetics Computer Group sequence analysis package.

responses to HIV-1 infection (6, 16). In particular, the well-characterized V3 region of *env* has been found to determine macrophage tropism (8, 16, 27, 32, 37). All of the V3 sequences obtained from the twins have extensive identity with sequences of macrophage-tropic HIV-1 isolates from adults. However, the sequences of some isolates from twins A and B, which display differences in macrophage tropism, have identical V3 sequences (for example, A10 and B1 [Fig. 3]), suggesting that other viral sequences besides V3 may contribute to macrophage tropism (4, 15, 41). The net charge of the V3 region has also been correlated with the SI or NSI phenotype (14). Based on the overall charge of the V3 loop sequence, all our sequenced isolates display a relatively low overall charge, which corresponded well with the observations that a low overall charge in the V3 loop correlates with the NSI phenotype (14). However, it is also possible that only a few SI virus particles may exist at the different time points, insufficient to produce an SI phenotype in our assay, and the cloning of viral sequences may have missed these minor populations so that their sequences are not reflected in our analyses.

Studies of natural HIV-1 transmission have shown that the recipients initially have a relatively homogeneous HIV-1 population, while the transmitters have a mixture of different viruses with a spectrum of genotypes and phenotypes (29). At later times after infection, severalfold-greater diversity was noted than at earlier stages of infection (28), suggesting that

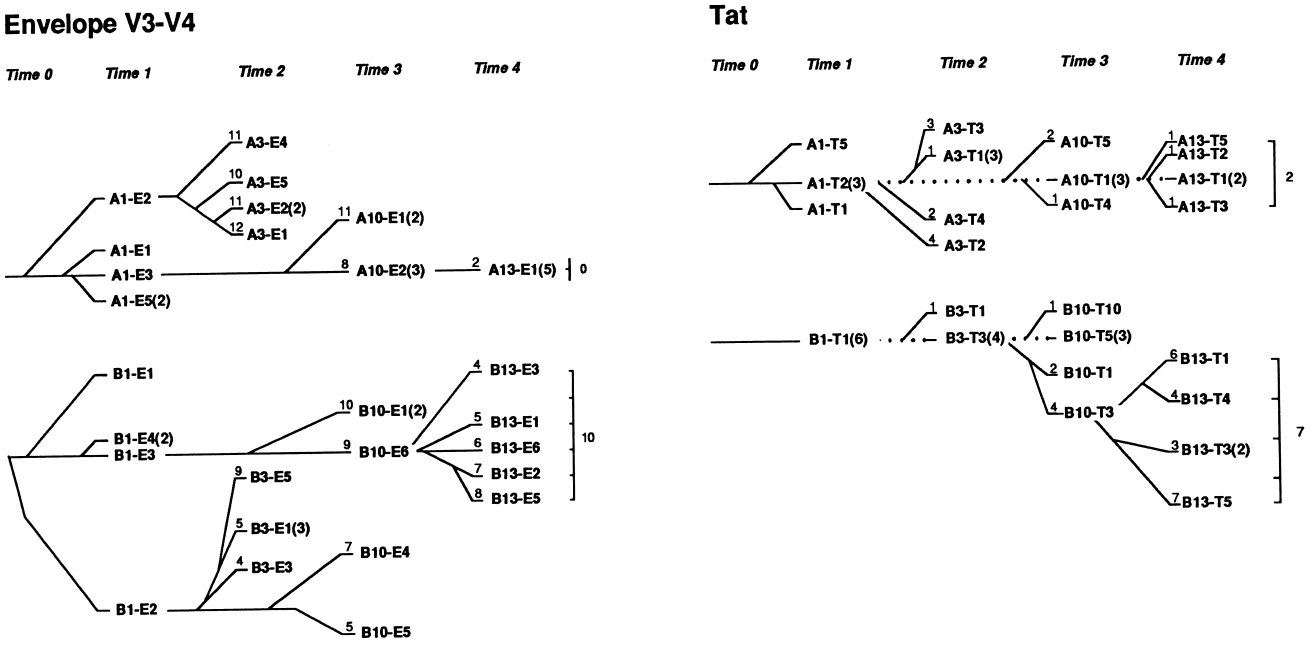

FIG. 6. Evolution over time of the viral *env* and *tat* gene sequences among the sequential isolates from the twins. Viral forms are designated by sequence number (Fig. 3). Evolutionary heritage (solid lines), with number of nucleotide changes from the earlier form noted above the terminus, evolutionary heritage with no nucleotide changes (dotted lines), and additional viral forms identical to the one designated (numbers in parentheses) are shown. The vertical spread of forms at a given time point represents the internal diversity among forms according to the scale of nucleotide differences provided at the extreme right. Time points correspond to successive sampling times for genetic analysis, with time 0 being a hypothetical time before which no sequence divergence had occurred.

sequence diversity may correlate with disease progression (22). Our results showed that diversity among viral forms increased to a greater extent at the final time point for the twin who progressed to AIDS than in the twin who did not. However, the degree of divergence as represented by nucleotide distances in our study should be met with some skepticism: it is based on a small sample size of cloned viral isolates which may not represent what may be significant to pathogenesis in vivo. Since the initial viral load cannot be determined, it will be impossible to distinguish whether the greater viral sequence diversity occurred in the sicker child because immune suppression allowed the emergence of genetic variants or whether the increased heterogeneity is generated by a high initial viral load in the sicker child's PBMC, leading to detection of additional minor viral populations. In contrast, a study by Delwart et al. (10) found a high degree of quasispecies complexity in the *env* gene of virus from asymptomatic adults with a strong immune response. These differences could be due to the above-mentioned limitations of sampling or could be the results of dissimilar patient populations. Their study contrasted fast disease progressors with slower progressors.

The possibility of PCR contamination in our sequence analyses cannot be eliminated, especially in a situation in which the patients have a shared epidemiology. PCR contamination among samples has indeed been of major concern recently (24). From our phylogenetic analysis, identical viral forms were not found in the two children but were commonly found within one twin. Viral forms obtained from the first time point for twins A and B differed from each other by an average of 2.7% (range, 2.2 to 3.7%) for the V3-V4 region and by 0.7% (range, 0.5 to 1.4%) for the *tat* region. These differences increased at later time points. The clustering of sequences from twins A and B within each twin suggests that there was no cross contamination, at least not between the twins. The phylogenetic analysis including V3-V4 sequences from both patients along with the consensus form of HIV-1 clade B also showed no significant clustering between the twins' sequences and clade B consensus sequences.

Despite the drawbacks due to sample availability and the limited number of viral isolates analyzed, our study with these heterozygotic twins represents a nearly ideal opportunity to monitor parallel viral phenotypic and genotypic changes and their relationships to change in the immune response and disease progression. It will still be important to examine the immune response as well as the viral *tat* and *env* gene sequences of twin A further to determine if sequence homogeneity is maintained as long as the child is healthy. Further studies using larger panels of longitudinal pediatric samples are also necessary in order to confirm our initial findings.

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