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Frequent Detection of Escape from Cytotoxic T-Lymphocyte Recognition in Perinatal Human Immunodeficiency Virus (HIV) Type 1 Transmission: the Ariel Project for the Prevention of Transmission of HIV from Mother to Infant

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Host immunologic factors, including human immunodeficiency virus (HIV)-specific cytotoxic T lymphocytes (CTL), are thought to contribute to the control of HIV type 1 (HIV-1) replication and thus delay disease progression in infected individuals. Host immunologic factors are also likely to influence perinatal transmission of HIV-1 from infected mother to infant. In this study, the potential role of CTL in modulating HIV-1 transmission from mother to infant was examined in 11 HIV-1-infected mothers, 3 of whom transmitted virus to their offspring. Frequencies of HIV-1-specific human leukocyte antigen class I-restricted CTL responses and viral epitope amino acid sequence variation were determined in the mothers and their infected infants. Maternal HIV-1-specific CTL clones were derived from each of the HIV-1-infected pregnant women. Amino acid substitutions within the targeted CTL epitopes were more frequently identified in transmitting mothers than in nontransmitting mothers, and immune escape from CTL recognition was detected in all three transmitting mothers but in only one of eight nontransmitting mothers. The majority of viral sequences obtained from the HIV-1-infected infant blood samples were susceptible to maternal CTL. These findings demonstrate that epitope amino acid sequence variation and escape from CTL recognition occur more frequently in mothers that transmit HIV-1 to their infants than in those who do not. However, the transmitted virus can be a CTL susceptible form, suggesting inadequate in vivo immune control.

The expanding human immunodeficiency virus type 1 (HIV-1) epidemic is fueled in part by perinatal transmission of the virus. Although antiviral therapy in the peripartum period significantly diminishes transmission (13), rates of 40% or higher occur in populations without ready access to pharmacological interventions (5, 47). The majority of these transmissions occur at or near the time of delivery (17, 18, 44). A better understanding of the factors contributing to perinatal transmission is key to designing new therapies that prevent transmission and is likely to provide insights regarding disease pathogenesis as well (45).

Perinatal HIV-1 transmission is typified by selection of a particular genotypic variant in the infected infant (46, 55). This selective virus transmission is compatible with several alternative hypotheses (61). First, the predominant viral sequence variant in the infant may be derived from an antigenically distinct variant in the mother that escaped the constraints of a critical immune surveillance mechanism. Second, transmission could be a stochastic phenomenon whereby the infants' viral sequences are acquired by random transmission of a limited number of virions or infected cells either at the time of birth or during gestation. While this genotypic variant may have rep-

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resented a major form in the mother at the time of transmission, this variant later could become a minor form in the mother because of genetic evolution. Lastly, the persistent form of virus in the infant may reflect differences in cell tropism, replicative capabilities, or chemokine coreceptor usage for viral entry (29, 43) that permit selective transmission or amplification of a particular genotypic variant within the infant (61).

Results from studies designed to elucidate the mechanism underlying perinatal HIV-1 transmission are conflicting. Prolonged exposure to infected cervicovaginal secretions is associated with transmission (41), but there are conflicting data regarding the relative contributions of high levels of virus in maternal peripheral blood (6, 11, 16, 19, 56, 58), levels of maternal neutralizing antibodies (14, 30, 34, 52, 53), and the ability to secrete inflammatory cytokines in response to viral peptides (9). Host immunologic factors have also been proposed as a factor in perinatal HIV-1 transmission and could possibly explain the observed selective transmission (54). The infant's viruses may be derived from an antigenically distinct variant arising in the mother that escapes a critical immune surveillance mechanism, possibly as a consequence of amino acid changes in B-cell (1, 23) or cytotoxic T-lymphocyte (CTL) epitopes (48, 59) that abrogate or antagonize immune recognition. These escape variants would, therefore, have a survival advantage that might facilitate their transmission.

Emerging data indicate that CTL exert significant immune pressure in HIV-1 infection, suggesting that this response might participate in modulating transmission. A temporal association between the emergence of HIV-1-specific CTL and a drop in plasma viremia has been observed during primary infection (3, 39), and strong CTL responses are maintained in persons with long-term nonprogressing HIV-1 infection and low viral loads (27, 35, 51, 60). CTL responses have also been associated with the emergence of immune escape due to mutations within CTL epitopes, both during primary infection (4) and with disease progression (24, 60), although the numbers of subjects studied has been small, and appropriate controls have been difficult to identify. The role of immune escape in disease pathogenesis remains controversial, as other studies have failed to identify escape as a means of disease progression (28). Although a vigorous human leukocyte antigen (HLA) class I-restricted immune response has been associated with a low rate of development of disease and increased time of survival, it is not clear why this host defense is ultimately unable to eliminate the virus and prevent disease progression. Within the infected host, sequestration of viruses in immunologically privileged sites, lack of adequate T helper cell function (8), down modulation of major histocompatibility complex (MHC) antigen expression (12), and amino acid sequence variation in well-defined class I-restricted CTL epitopes (48, 59) have been postulated to explain this apparent paradox (63). Selection for a virus population harboring amino acid sequence changes in residues within or near class I-restricted epitopes could facilitate CTL escape by disrupting correct endogenous processing of the antigen, HLA binding, and/or optimal recognition of the peptide-HLA complex by T-cell receptors (48).

Whether transmission per se is associated with escape from CTL recognition has yet to be determined conclusively. We reasoned that the question of whether CTL serve as an important immunological defense could be addressed experimentally in a setting in which transmission could be readily monitored and in which the transmitted virus could be examined. We therefore evaluated the maternal HIV-1-specific CTL responses in a cohort of transmitting and nontransmitting mothers (6). Mothers enrolled in the Ariel Cohort for the Prevention of HIV Transmission from Mother to Infant were evaluated for the epitope specificity of their clonal CTL responses at the time of delivery. Additionally, autologous viral sequences were amplified, at endpoint dilution, from peripheral blood obtained from each HIV-1-infected mother and her infected infant at or within days of the time of delivery to screen for evidence of sequence variation within the defined CTL epitopes and to determine the effects of such amino acid sequence changes on recognition by maternal CTL. Finally, we evaluated the virus obtained from the infants for recognition by the maternal CTL response as a correlate of immune escape and a potential mechanism underlying perinatal HIV-1 transmission.

MATERIALS AND METHODS

Subjects. Study subjects were selected from a cohort of HIV-1-infected pregnant women enrolled in the Ariel Project for the Prevention of Transmission of HIV from Mother to Infant and monitored throughout their pregnancies. The Ariel cohort enrolled mothers at the seven clinical sites located in Fort Lauderdale, Fla.; Newark, N.J.; Houston, Tex.; Bronx, N.Y.; Stamford, Conn.; Worcester, Mass.; and New Orleans, La. Subjects included in this study were prospectively monitored at the Houston, Bronx, Worcester, and Fort Lauderdale clinical sites. The characteristics of the Ariel cohort are described elsewhere (6). Among the total of 204 HIV-1-infected pregnant women enrolled in the study, we selected 3 of the 8 women who infected their infants during gestation and 8 of the 185 women who did not, based on sample availability and the detection of an immunodominant CTL response. The numbers of copies of virion-associated RNA per milliliter of plasma were quantified for each woman at the time peripheral blood was obtained for T-cell cloning, a visit corresponding to the time of delivery, by the AMPLICOR HIV-1 Monitor Test (Roche Diagnostic Systems, Inc., Branchburg, N.J.) as instructed by the manufacturer. The infection status of the infants born to these mothers was determined by the presence of HIV-1 proviral DNA in peripheral blood mononuclear cells by using a PCR-based assay and by viral coculture at delivery and at regular intervals thereafter.

Cell lines. Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were established from the peripheral blood mononuclear cells (PBMC) of each subject and maintained as previously described (57) in RPMI 1640 medium (Sigma, St. Louis, Mo.) containing 20% heat-inactivated fetal calf serum (Sigma). RPMI 1640 medium used for all cell lines was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and HEPES (10 mM). Additional allogeneic B-LCL used in HLA restriction experiments were established and maintained in a similar fashion.

HLA class I typing. HLA typing was performed by using a standard lymphocytotoxicity assay and confirmed in some cases with a PCR-based allele-specific molecular typing assay (24).

Recombinant vaccinia virus constructs. Recombinant vaccinia viruses expressing the full-length HIV-1 gp160 gene (VPE16) as well as serial truncations of the HIV-1 envelope gene (VPE17-22) were provided by P. Earl and B. Moss. Recombinant vaccinia viruses expressing HIV-1 $p55^{gag}$ (vAbT141), $p24^{gag}$ (vAbT286), $p17^{gag}$ (vAbT228), HIV-1 Pol (vAbT204), HIV-1 Nef (vT23), and wild-type control (NYCBH) were provided by Therion Biologics (Cambridge, Mass.). Stocks of recombinant vaccinia viruses were adjusted to approximately 10^9 PFU/ml, stored in aliquots at −80°C, and thawed immediately prior to use.

HIV-1 peptide synthesis. Synthetic peptides corresponding to the HIV-1 HXB10 sequence (26), consisting of a series of peptides 25 amino acids in length that overlapped by 8 amino acids, were synthesized and purified by Multiple Peptide Systems (San Diego, Calif.) as described previously (31). Peptides were synthesized as COOH-terminal amides unless otherwise noted. Smaller peptides of 8 to 15 amino acids used for fine mapping were synthesized as free acids on an automated peptide sequencer (Applied Biosystems model 420A). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethyl sulfoxide (Sigma) and 1% 1 mM dithiothreitol (Sigma).

Isolation of HIV-1-specific CTL clones and lines. CTL clones were isolated and maintained as described previously (57). Briefly, PBMC were obtained by separation of whole blood on a Ficoll-sodium diatrozoate gradient (Sigma) and were plated at concentrations ranging from 10 to 100 cells per well of a 96-well plate. Cells were maintained with a feeder solution containing 10^6 irradiated allogeneic PBMC per ml from uninfected subjects in RPMI 1640 with 10% heat-inactivated fetal calf serum supplemented with 100 U of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, N.J.) per ml. The CD3-specific monoclonal antibody (MAb) 12F6 (62) was added at a final concentration of 0.1 μg/ml to stimulate T-cell proliferation. Cells from wells demonstrating growth were restimulated further as previously described (32) and then tested for cytolytic activity against autologous target cells infected with recombinant vaccinia viruses expressing HIV-1 genes approximately 4 to 6 weeks after the initial cloning. T-cell clones exhibiting HIV-1-specific CTL activities were restimulated every 14 to 21 days with anti-CD3 MAb and irradiated allogeneic PBMC.

TABLE 1. Characteristics of mothers

Subject	Transmission status[a]	CDC stage[b]	% CD4	CD4 cells/ml	Viral RNA (mol/ml)	AZT status[c]
11113	T, late	A1	21	639	11,000	I
19142	T, early	A1	43	645	7,700	N
19145	T, late	A2	21	466	200	N
02113	NT	B3	8	90	11,000	I
08106	NT	A2	23	267	2,300	C
08107	NT	A2	19	325	5,900	N
08109	NT	A1	29	555	510	N
08118	NT	A2	39	403	200	N
19102	NT	A3	29	180	2,800	N
19103	NT	A2	28	386	680	I
19143	NT	B3	14	130	36,000	C

[a] NT, mother was a nontransmitter; T, mother was a transmitter; early, child had detectable virus within 24 h of birth, by PCR detection of viral DNA in the child's PBMC, viral RNA in the plasma, and/or PBMC in viral culture; late, child was HIV negative on the first day of life but was HIV positive by the second blood draw at 1 month of age.

[b] Includes $CD4^+$ T-lymphocyte and clinical categories and is based on the 1993 Revised Classification for HIV Infection and Expanded Surveillance Case Definition for AIDS among Adolescents and Adults (7).

[c] N, no AZT was given during the pregnancy or birth; C, mother had initiated AZT use prior to pregnancy and was continuing to use AZT throughout pregnancy and delivery; I, mother initiated AZT use during pregnancy, following through with a regimen based on the initial recommendations of the 076 study, the study that first showed the benefits of AZT use in reducing perinatal transmission (13).

Flow cytometric analysis. Cells were incubated with fluorescent probe-conjugated anti-CD3/anti-CD4, anti-CD3/anti-CD8, and anti-mouse immunoglobulin G2b (IgG2b)/IgG1 MAbs as controls (Coulter Electronics, Hialeah, Fla.), singly or in combination. Samples of stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, Calif.) as described previously (32).

Cytotoxicity assay. Target cells consisted of B-LCL infected with recombinant vaccinia viruses or preincubated with synthetic HIV-1 peptides. Target B-LCL were infected with recombinant vaccinia virus as previously described (59), and labeled with 65 to 100 μCi of [$^{51}CrO_4$] Na_2 (New England Nuclear, North Billerica, Mass.) overnight, and then washed three times with RPMI 1640 medium. Peptide-sensitized target cells were obtained by incubating 2×10^6 to 3×10^6 B-LCL with peptide for 60 min during ^{51}Cr labeling. Cytolytic activity was determined in a standard 4-h ^{51}Cr release assay using U-bottom microtiter plates containing 5×10^3 targets per well. Assays were performed in duplicate. Supernatant fluids were harvested onto 96-well plates containing solid scintillate, allowed to dry overnight, and then counted in a TopCount microplate scintillation counter (Packard Instrument Co., Meriden, Conn.). Maximum release was determined by lysis of targets in detergent (1% Triton X-100; Sigma). Percent lysis was determined as $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release values were less than 30% of maximal release for all reported assays.

In vitro amplification of epitope-encoding regions and viral sequence analysis. To explore the relationship between CTL recognition or escape and genetic diversity within class I MHC-restricted epitopes, we assessed the diversity of viral sequences within these individuals by examining proviral sequences spanning selected fragments of the *gag*, *pol*, *env*, and *nef* coding regions. PCR was used to amplify proviral DNA at endpoint dilution in cells obtained from the index visit near the time of parturition. In some cases only short fragments spanning just the regions of interest were sequenced, but in other cases longer sequences were generated, and those sequences were incorporated into the phylogenetic analysis shown in Fig. 3. The positions of the oligonucleotide primers are numbered according to the HXB2 isolate in the human retroviruses and AIDS database (37). LTRF1.1 (nucleotides 518 to 542; 5′-TAAGCCTCAATAAAGCTTGCCTTG-3′), LTRF2.1 (nucleotides 574 to 542; 5′-TGTGACTCTGGTA[A/G]CTAGAGATCCC-3′), LTRF3.1 (nucleotides 626 to 650; 5′-TCTCTAGCAGTGCGCCCCGAACAGG-3′), VACGAGR1 (nucleotides 2314 to 2338; 5′-TCTGCTCCTGTATCTAATAGAGCTT-3′), VACGAGR2 (nucleotides 2375 to 2399; 5′-TCC[C/T]CCTATCATTTTTGGTTTCCAT-3′), VACGAGR3 (nucleotides 2468 to 2492; 5′-TGTAGGTCCTACTAATACTGTACCT-3′), and CTLPOLF1 (nucleotides 2318 to 2341; 5′-TCTATTAGATACAGGAGCAGATGA-3′) are the *gag* amplification primers. The outer sets of *gag* amplification primers and their amplicon sizes are LTRF1.1 and VACGAGR3 (1,974 bp), LTRF1.1 and VACGAGR2 (1,881 bp), LTRF2.1 and VACGAGR3 (1,918 bp), and LTRF2.1 and VACGAGR2 (1,825 bp). The inner sets of *gag* amplification primers and their amplicon sizes are LTRF2.1 and VACGAGR2 (1,825 bp), LTRF2.1 and VACGAG1 (1,764 bp), LTRF3.1 and VACGAGR1 (1,712 bp), and LTRF3.1 and VACGAGR2 (1,974 bp). CTLPOLF2 (nucleotides 2391 to 2415; 5′-TAGG[G/A]GGAATTGGAGGTTTTATCA-3′), CTLPOLF3 (nucleotides 2467 to 2490; 5′-TAGGTACAGTATTAGTAGGACCTA-3′), CTLPOLR1 (nucleotides 4060 to 4083; 5′-TATCTGGTTGTGCTTGAATGATTC-3′), CTLPOLR2 (nucleotides 4321 to 4344; 5′-TGGCTACTATTTCTTTTGCTACTA-3′), and CTL POLR3 (nucleotides 4649 to 4673; 5′-TTGACTTTGGGGATTGTAGGGAAT-3′) are the *pol* amplification primers. The outer sets of *pol* amplification primers and their amplicon sizes are CTLPOLF1 and CTLPOLR3 (2,355 bp), CTLP OLF1 and CTLPOLR2 (2,355 bp), CTLPOLF2 and CTLPOLR3 (2,282 bp), and CTLPOLF2 and CTLPOLR2 (1,953 bp). The outer sets of *pol* amplification primers and their amplicon sizes are CTLPOLF2 and CTLPOLR2 (1,953 bp), CTLPOLF2 and CTLPOLR2 (1,953 bp), CTLPOLF2 and CTLPOLR1 (1,692 bp), CTLPOLF3 and CTLPOLR2 (1,877 bp), and CTLPOLF3 and CTLPOLR1 (1,616 bp). KKE5P3 (nucleotides 6189 to 6213; 5′-GCGACCCGGGTTGATAGA[C/A]TAA[T/G]AGAAAGAGCAGA-3′) and KKE3P1 (nucleotides 8809 to 8833; 5′-GCGAGAATTCATCC[C/A]AC[T/C]A[C/T]ACTAC[T/G]TTTTGACCA-3′) are the *env* amplification primers. The input DNA molecules were quantified by PCR using serial fivefold dilutions. Twenty 5-μl samples of endpoint-diluted DNA were amplified in a 100-μl reaction mix containing 0.2 μM outer primer pair as described elsewhere (22). PCR was performed with a Perkin-Elmer/Cetus 9600 automated thermal cycler programmed for for 35 cycles at 98°C for 10 s, 50°C for 30 s, and 72°C for 3 min, with a final extension at 72°C for 10 min. A 5-μl sample was reamplified from each reaction in a 100-μl reaction mix containing 0.2 μM inner primer pair by means of the same cycle profile as specified above. HIV-1-negative cell DNA and reagent controls were run in parallel (40). PCR product DNA was resolved by electrophoresis on a 1.0% NuSieve GTG gel (FMC BioProducts). The correctly sized band was purified from the agarose gel by electroelution using gene capsules (Geno Technologies, Inc.) and inserted into pCR 2.1 (Invitrogen), using the principles of TA cloning. One microgram of the double-stranded DNA template was sequenced in both forward and reverse directions, using the respective *gag*, *pol*, and *env* internal primers with the use of dideoxynucleoside triphosphates (Dye-Deoxy terminators) and analyzed with a model 377 sequencing system (Applied Biosystems) as described elsewhere (22).

Phylogenetic reconstruction and statistical analysis. The sequences were hand aligned by using a modified version of the MASE program (20). The sequences were gap stripped (42), and phylogenetic analysis was done by the neighbor-joining, bootstrap, and maximum-likelihood methods from the PHYLIP package (21). Statistics were done with the Splus package, version 3.4 (MathSoft, Inc.).

Nucleotide sequence accession numbers. The viral sequences reported in this study are available in GenBank, under accession no. AF121459 to AF121669.

RESULTS

Characterization of the maternal CTL response. Subjects from the Ariel cohort were randomly screened for CTL responses to HIV at or near the time of delivery. Three HIV-1-infected mothers who infected their infants and eight mothers who did not transmit virus were selected for detailed study on the basis of sample availability and the ability of their established T-cell clones to recognize HIV-1-specific antigens. The ranges of viral loads measured at delivery among the two groups were not statistically distinguishable, in accord with the small sample size. There was also no discernible difference between the two groups in terms of intrapartum azidothymi-

TABLE 2. CTL target epitopes in transmitting mothers and their infants

Mother-infant pair	Location[a]	Tree[b]	HLA	Mother			Infant		
				Sequence[c]	CTL[d]	Frequency[e]	Sequence	CTL	Frequency
11113-11213	p17 (18–26)	GagA	A3	KIRLRPGGK	++	1/12	KIRLRPGGK	++	11/11
				--------R	−	10/12			
				R-------R	−	1/12			
	p24 (298–306)	GagB	B14	DRFYKTLRA	++	2/17	DRFYKTLRA	++	10/11
				-----I---	−	15/17			
							-Q-------	−	1/11
	Pol (325–333)	PolA	A3	AIFQSSMTK	++	18/18	AIFQSSMTK	++	13/17
							--------R	++	1/17
							--L------	NT	1/17
							---L-----	−	1/17
							T-S------	−	1/17
19142-19242	gp120 (375–383)		B15[f]	SFTCGGEFF	++	6/9	SFTCGGEFF	++	5/5
				-S-------	+	1/9			
				------GV-	−	1/9			
				------G--	+	1/9			
	gp41 (557–565)		B15[f]	RAIEAQQHL	++	7/7	RAIEAQQHL	++	5/5
	Nef (120–128)		B15[f]	FFPDWKNYT	+	10/10	FFPDWKNYT	+	9/10
							L--------	−	1/10
19145-19245	Pol (438–446)		B42	YPGIKVRQL	++	2/9			
				------K--	++	3/9			
				-HK------	NT	1/9			
				-H-------	−	1/9			
				-A-------	++	2/9	YAGIKVRQL	++	10/10

[a] The epitope boundaries (in parentheses) are written with respect to the reference strain IIIB.
[b] Subset of epitopes that were sequenced in the context of long fragments that were subjected to phylogenetic analysis, the results of which are shown in Fig. 3. (Some of the epitope sequences were embedded in fragments that were too short to subject to further analysis, providing only enough information to span the epitope of interest.)
[c] If the same sequence spanning a particular epitope was found in the mother and the infant, it is written on the same horizontal line; all variant epitopes are aligned to the top one, and identical amino acids indicated with a dash.
[d] Intensity of the CTL response, shown graphically in Fig. 2, ++, strong CTL response; +, reduced CTL response, comparable to a half-maximal lysis that is 2 logs less than that of the index (or most reactive) peptide and that has an absolute specific lysis of >20% at two peptide concentrations; −, the T cell does not recognize the peptide, and the absolute specific lysis is >20% at either only one or at none of the peptide concentrations; NT, not tested.
[e] Number of each epitope sequence obtained and overall frequency.
[f] Shared by mother and infant.

dine (AZT) use, but those mothers who infected their infants in our study tended to have higher peripheral $CD4^+$ T-cell counts (Table 1).

We determined the dominant CTL responses, by limiting-dilution cloning using a CD3-specific MAb as a stimulus for T-cell proliferation (57). Expanded cell lines were tested for recognition of autologous B-LCL infected with recombinant vaccinia viruses expressing HIV-1 antigens (33). Cells with HIV-1-specific CTL activity were mapped in terms of epitope specificity by using synthetic peptides to sensitize autologous B-LCL, and HLA restriction was determined (Tables 2 and 3). The HLA haplotype was resolved by using a standard lymphocytotoxicity assay and in some cases confirmed by a PCR-based hybridization assay using anchored allele-specific oligonucleotide probes (24) that also confirmed the genetic linkage between infant and mother (data not shown).

Figure 1 shows representative examples of HIV-1-specific T-cell clones from a selected pair of transmitting (11113) and nontransmitting (19143) mothers. The two mothers exhibited similar plasma virion-associated RNA copy numbers and targeted the same HLA A3-restricted epitope, KIRLRPGGK, within the p17 region of Gag (27). T-cell clones from the two mothers were almost identical in the ability to recognize epitope-expressing target cells, as demonstrated by similar kill-

TABLE 3. CTL target epitopes in nontransmitting mothers

Mother	Location[a]	Tree[b]	HLA	Sequence[c]	CTL[d]	Frequency[e]
02113	Pol (591–600)	PolB	B45[f]	GVETFYVDGA	++	11/11
08106	Pol (593–603)	PolB	A26[f]	ETFYVDGAANR	++	12/15
				--Y--N-----	+	3/15
08107	p24 (180–188)	GagA	B7	TPQDLNTML	++	10/10
08109	gp120 (376–384)		A29	PNCGGEFFY	++	7/15
				---R-----	−	8/15
08118	p24 (324–335)	GagB	B51[f]	NANPDCKTI	++	11/11
19102	Pol (262–270)	PolA	B35	TVLDVGDAY	++	11/12
				----M---C	++	1/12
19103	p24 (273–283)	GagA	B52	RMYSPTSI	++	14/14
19143	p17 (18–26)	GagA	A3[f]	KIRLRPGGK	++	16/16

[a–f] See the footnotes to Table 2.

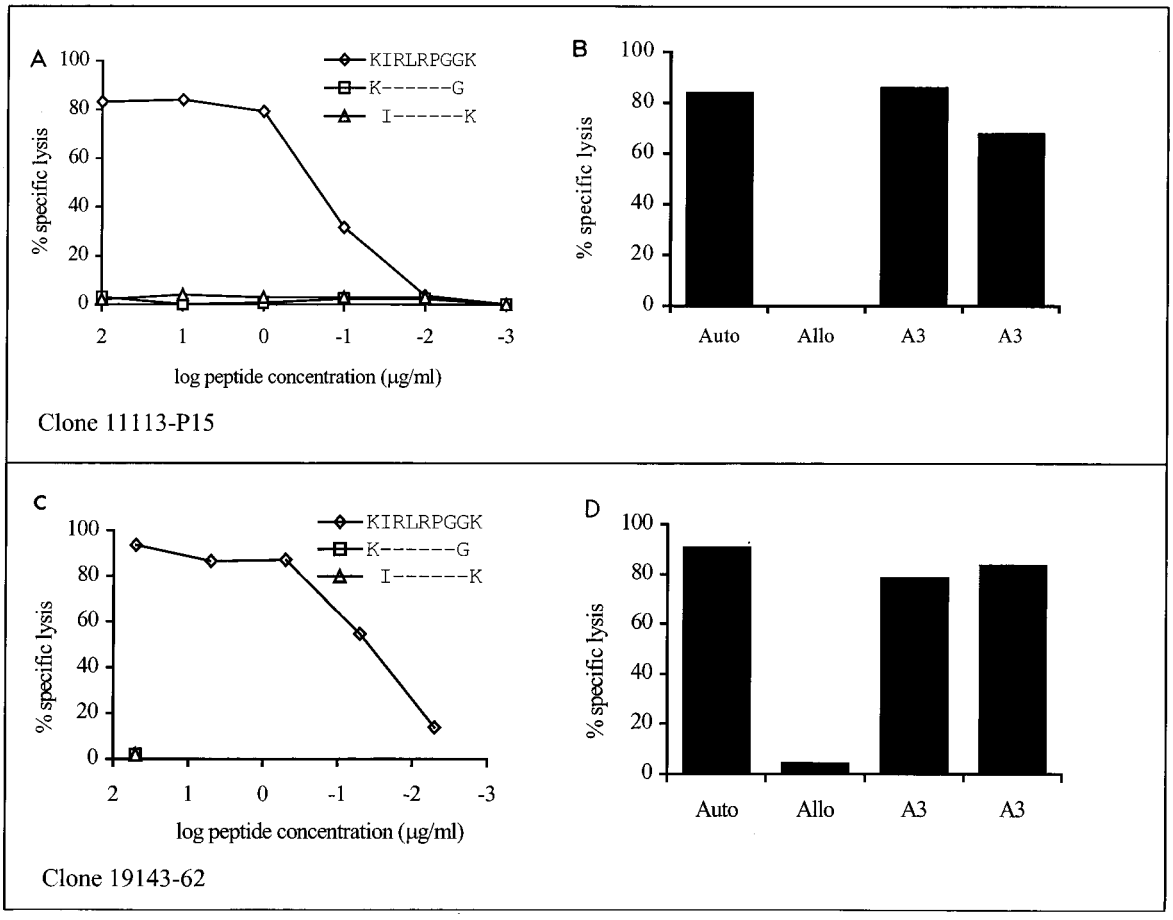

FIG. 1. Specificity of CTL clones derived from a transmitting and nontransmitting mother. (A and C) CTL clones derived as described in the text were mapped in terms of epitope recognition by using recombinant vaccinia viruses (data not shown) followed by peptide-sensitized target cells. The range of sensitizing peptide concentrations is shown. HLA restriction was determined by using B-LCL target cells infected with recombinant vaccinia viruses expressing Gag protein (B and D). Auto, the autologous B-LCL; Allo, an HLA-mismatched B-LCL; A3, two different allogeneic B-LCL matched only at HLA-A3. Effector-to-target ratios for each assay were 5:1.

ing profiles at limiting peptide concentration. The finding that a transmitting and a nontransmitting mother could target the same epitope suggests that epitope specificity alone is not likely to be a determining factor in transmission. We performed similar detailed studies using peripheral blood samples obtained from three transmitting mothers and eight nontransmitting mothers, the results of which are shown in Tables 2 and 3. A total of eight T-cell clones from nontransmitting mothers and seven clones from transmitting mothers were fully evaluated by these detailed methods. For two of the three transmitting mothers, multiple T-cell clones with distinct specificities were identified, whereas for all of the eight nontransmitters, CTL of only a single specificity were isolated (Tables 2 and 3). The association with detection of CTL responses to multiple epitopes (two of three versus none of eight) was of borderline significance (Fisher's exact test, $P = 0.0545$). Overall, HIV-1-specific responses to Gag, Env, Pol, and Nef were detected, each restricted by an identifiable HLA class I antigen. These data add to a growing number of well-defined HIV-1-specific CTL epitopes, and indicate that targeting of specific regions of the virus was not unique to T cells obtained from either mothers who infected their offspring or those who did not.

Genetic variation within targeted HLA class I-restricted epitopes. Having identified the HIV-1-specific epitopes targeted by the mothers, we next evaluated viral sequence variation within the specific HLA class I-restricted epitope and determined the effects of sequence variation on CTL recognition. We analyzed the viral sequences within each of the infected mothers by examining proviral sequences spanning the relevant portions of the *gag*, *pol*, *env*, and *nef* coding regions of the viral genome from peripheral blood samples obtained at the time of delivery. The target sequences were amplified by nested PCR at endpoint dilution to obviate resampling of any single template molecule (38). Product DNAs were inserted into pAMP by UDG cloning, and 5 to 17 clones from each sample were sequenced in both directions. Several of the epitopes were sequenced only across a short fragment, spanning the epitope, while others were sequenced embedded in long sequences, augmented by additional short fragments. Those epitope sequences that were obtained embedded in longer fragments were suitable for phylogenetic analysis. In all cases where longer sequences were available, phylogenetic reconstructions of the mothers' viral sequences showed distinct clusters corresponding to the respective nontransmitting mother, or mother-infant pair, indicating absence of PCR-product contamination and establishing epidemiological linkage between the transmitting mother and her infant, and proving that there were no sample mix-ups (see Fig. 3). BLAST

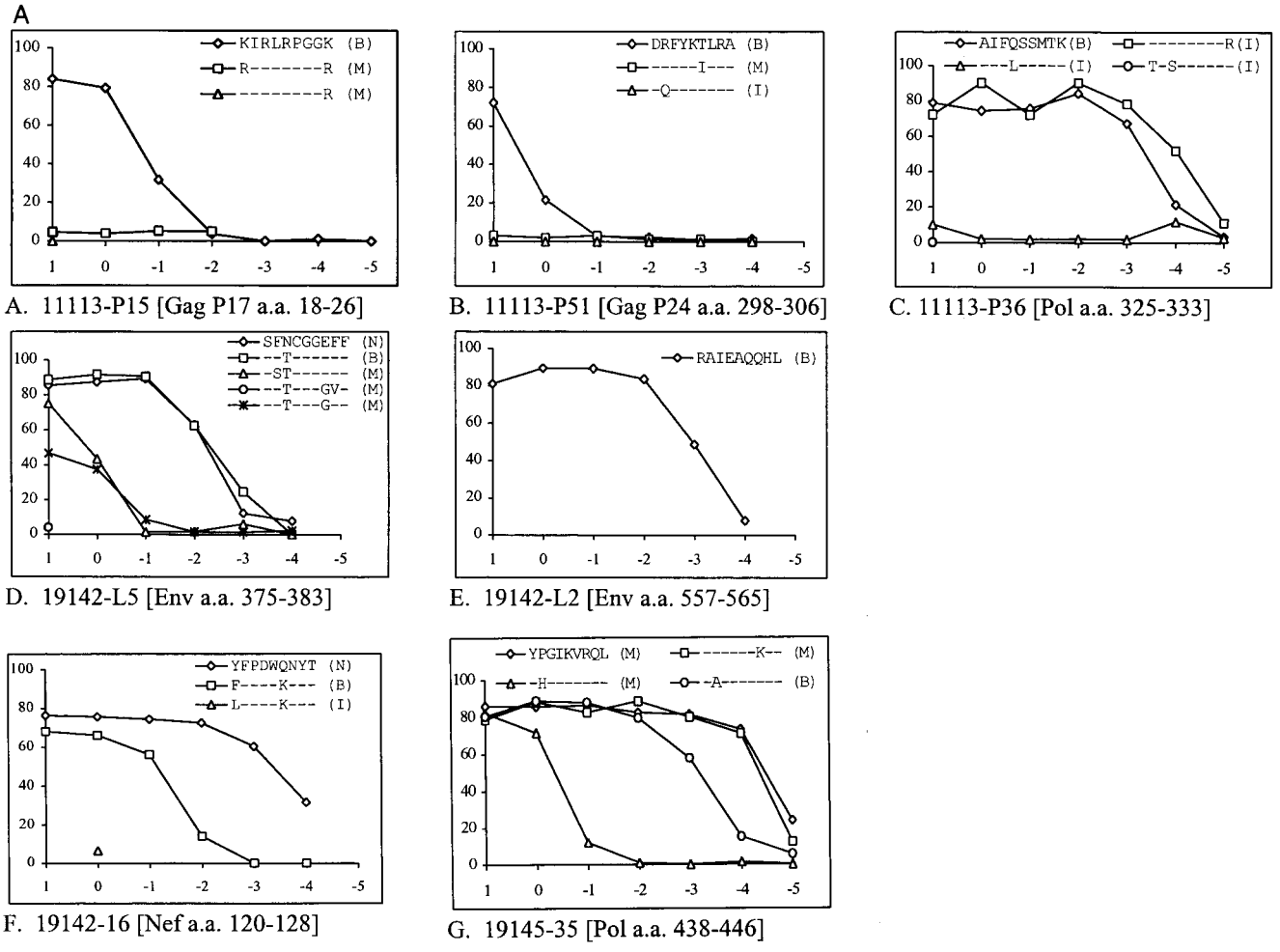

FIG. 2. Specificity of CTL clones derived from transmitting (A) and nontransmitting (B) mothers, including recognition of in vivo sequence variants. CTL clones were tested for recognition of target cells sensitized with peptides representing in vivo virus variants, and values are given on the *y* axis as percent specific lysis. Peptides were tested over a range of concentrations as indicated on the *x* axis (log peptide concentrations are listed in micrograms per milliliter). For each peptide, the lowest concentration of peptide which resulted in lysis was determined. For transmitting mothers, CTL were also tested for recognition of the variants present in the first infant isolate. B, sequence detected in both the mother and infant; I, sequence detected in only the infant; M, sequence detected in only the mother; N, HIV-1 IIIB sequence to which the epitope mapped but which was not detected in either the mother or infant. Effector-to-target ratios for each assay were 5:1.

searches against GenBank gave no indication of any contamination problems.

The extent of genetic diversity within an A3-restricted p17 epitope differed for one mother who infected her infant (11113) compared to another who did not (19143) (Tables 2 and 3). While the A3-restricted p17 epitope sequence KIRLRPGGK was highly conserved among the genetic variants obtained from nontransmitting mother 19143, amino acid substitutions were found within the same A3-restricted p17 epitope for transmitting mother 11113. The predominant epitope sequence had a lysine-to-arginine (K-to-R) substitution at the carboxyl terminus, an important residue for peptide binding to A3 (15, 50), and was not recognized by the CTL from transmitting mother 11113 (Fig. 2) or by the CTL from nontransmitting mother 19143 (data not shown). Likewise, among the other transmitting mothers 19142 and 19145, a high proportion of epitope variants were found within at least one of the epitopes targeted. Overall, four of the seven defined HLA class I-restricted epitopes targeted by T-cell clones obtained from transmitters 11113, 19142, and 19145 had amino acid changes, with a range of two to five variant forms of the epitope detected. Among the eight nontransmitting mothers, three of the eight defined HLA class I-restricted epitopes targeted by T-cell clones had amino acid changes, and there were never more than two variant forms present.

The relationship between epitope variation and escape from maternal HLA class I-restricted CTL recognition was investigated by synthesizing peptides corresponding to each of the variant epitopes and testing their ability to be recognized by the maternal T-cell clones at limiting peptide concentration (27). Based on our prior studies of escape from CTL detection, we defined a variant peptide to be an immune escape variant if it required a concentration 2 logs higher than the canonical epitope sequence to effect half-maximal lysis. Epitope mapping studies showed that CTL clones derived from nontransmitting mother 19143 recognized the only detected A3-restricted P17 epitope (KIRLRPGGK), whereas the CTL clone from transmitter 11113 did not recognize the autologous variants of the same A3-restricted epitope (Fig. 2). Among all eight nontransmitting mothers, the CTL clone derived from mother 08109 was the only one that failed to recognize one of its autologous viral variant epitope sequences (A29-restricted gp120 epitope

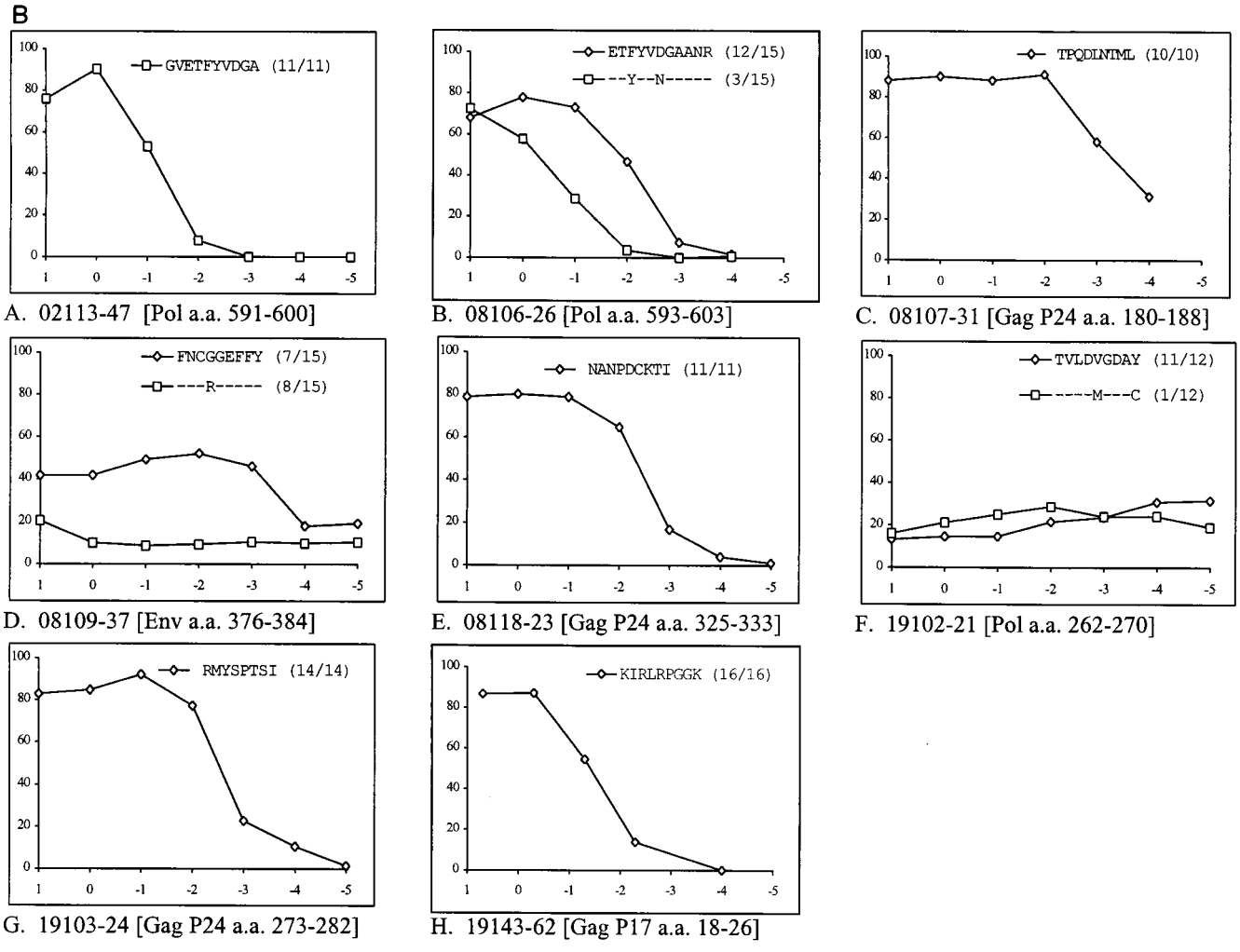

FIG 2—*Continued*.

[Table 3 and Fig. 2B). In contrast, all three of the transmitting mothers showed evidence of immune escape, failing to recognize the autologous peptides for four of the seven defined T cell epitopes (Table 2 and Fig. 2A). For transmitting mother 11113, the predominant in vivo viral variants in the A3-restricted p17 epitope (KIRLRPGG**R**) and the B14-restricted p24 epitope (DRFYK**I**LRA) represent immune escape sequences, whereas immune escape variants were rare but present in transmitting mothers 19142 and 19145. That three of three transmitters, and only one of eight nontransmitters carried escape variants was statistically significant (Fisher's exact test, $P = 0.0242$). This analysis, using the 2-log definition of an escape variant, suggests that transmitters have a higher propensity to carry escape variants than nontransmitters but does not account for different degrees of CTL recognition of variant peptides. An even more dramatic way to consider the distinction between transmitters and nontransmitters is to consider the number of highly recognized CTL epitopes, those that have reduced recognition, and those that are unrecognized (++, +, and −, respectively, in Tables 2 and 3, based on Fig. 2). Based on a tally of observed variant sequences and CTL reactivity, the transmitting mothers have 41 ++ reactive epitopes, 12 +, and 28 −, while the nontransmitting mothers have 93 ++, 3 +, and 8 −. A 3×2 contingency table indicates that there is a low probability that such distinctive sets of observed variants could have occurred by chance alone ($P \ll 10^{-4}$).

Maternal CTL recognition of transmitted viruses. We next sought to determine if CTL clones derived from the mother recognized the defined HLA class I-restricted cognate epitope in the infant's sequences. Peptides corresponding to the variant epitopes found in the infants were synthesized and then tested for recognition by their mother's CTL clones. Two of the three infants had defined epitope sequences with mutations that conferred escape from maternal CTL recognition, although in all three infants the most prevalent epitope sequences were CTL susceptible (Table 2 and Fig. 2A). Interestingly, none of the infant genetic escape variants was found in the mother at the time of peripheral blood sampling. Furthermore, although in several cases CTL escape variants were the predominant sequences isolated in the mother, those escape sequences were not detected in the respective infants (Table 2 and Fig. 2A). Instead, a minor maternal variant sequence, well recognized by maternal CTL, was often the major epitope sequence isolated in the infant. On the whole, more than 90% of the infant epitope sequences tested were recognized by maternal CTL.

One possible explanation for the presence of CTL-susceptible virus in the infant is that reversion to a more fit virus may have occurred in vivo in the infant in the absence of immune

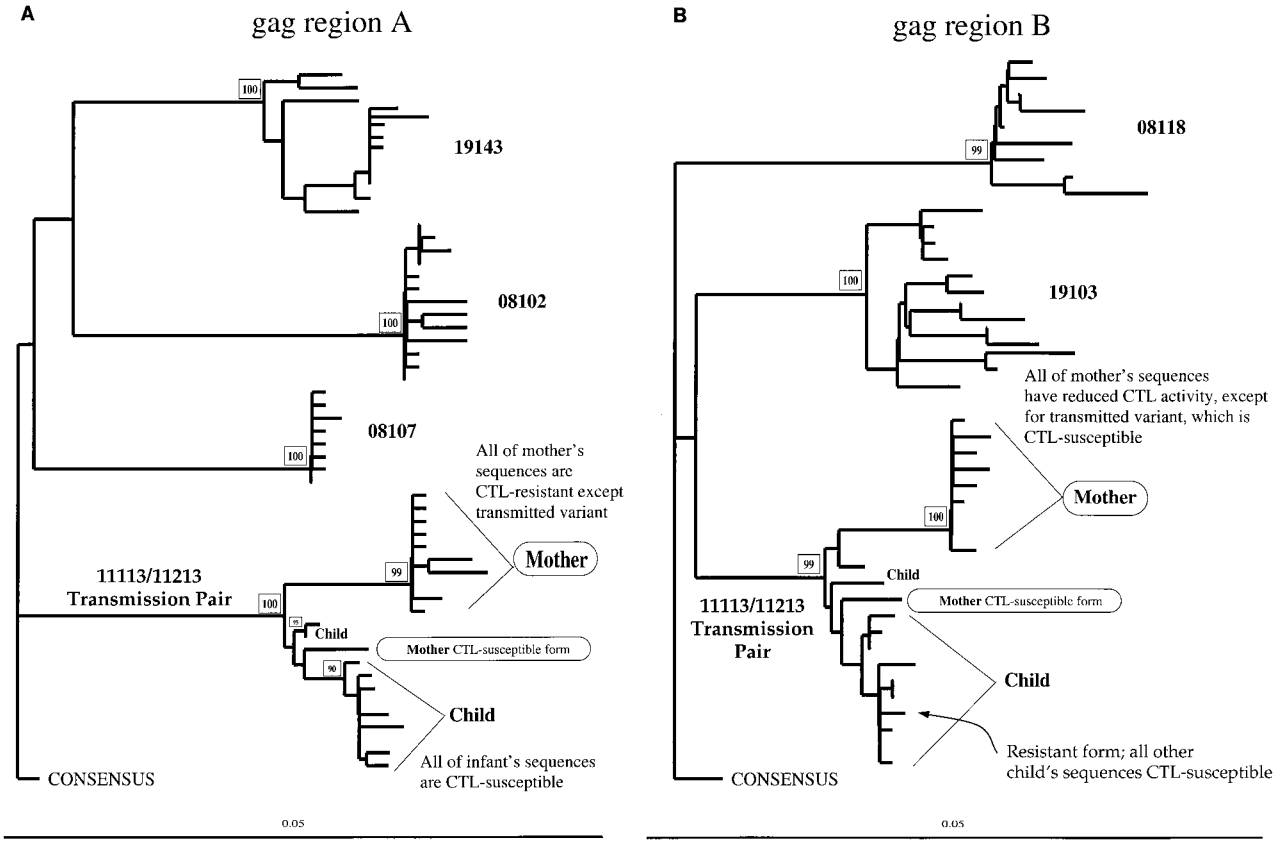

FIG. 3. Phylogenetic reconstruction of viral sequences from peripheral blood. The trees shown were generated by the neighbor-joining method using the maximum-likelihood method for calculating distances in the options provided by the neighbor program in the PHYLIP package (21). Neighbor-joining bootstrap values of >50 that were generated with PHYLIP are also shown as boxed numbers. A distance scale is shown along the bottom of each panel. Maximum-likelihood trees generated with DNA supported the phylogenetic relationships noted, particularly the intrapatient associations and the clustering of the mother's susceptible form with the infant's sequences in the two Gag trees. The long fragments with epitopes embedded in them were used to construct the trees; the epitopes included within the fragments are indicated in Table 1. (A) GagA tree, including 57 sequences and (857 positions); panel (B) GagB tree, including 45 sequences (876 positions). Of note is the integrity of the sequences; each patient has a clear cluster of sequences. PolA (including 59 sequences [434 positions]) and PolB (including 55 sequences [671 positions]) trees were also generated and showed similar sequence integrity (data not shown). In the GagA and GagB trees, one can see that the rare CTL-susceptible variant from transmitting mother 11113 is actually the form that clusters with the virus from infant 11213. CTL susceptibility is based on the epitope fragments shown in Table 1.

pressure. To address this issue, phylogenetic analysis was performed on one of the mother-infant pairs in which adequately long sequences for careful analysis could be obtained. The clustering patterns of the sequences from the mother-infant pair (mother 11113 and infant 11213) indicate that the unusual, CTL-susceptible form found in the mother was actually the transmitted variant of the virus, rather than that the backmutation to wild type from a CTL-resistant form occurred in the infant (Fig. 3B). Thus, although CTL escape was significantly associated with transmission, the actual transmitted virus was apparently not an escape variant.

DISCUSSION

This study is unique in providing a detailed molecular analysis of the HIV-1-specific epitopes targeted by the CTL response in mothers who transmitted the virus to their offspring, in their infected infants, and in HIV-1-infected mothers who did not infect their offspring. Within the limitations of our data set, we found an association between greater viral sequence variation in well-defined, HLA class I-restricted maternal epitopes and the propensity for the mother to transmit the virus during gestation. These results further indicate that viral escape from CTL recognition is frequently detected in mothers who transmit virus to their infants and that minor susceptible variants in the mother can become the major variant transmitted to the infant. In contrast to the immune escape detected in the transmitting mothers, lack of CTL recognition of in vivo virus was infrequently detected in nontransmitting mothers. Four of the seven CTL clones derived from the three mothers who transmitted virus to their offspring, and only one CTL clone derived from a mother who did not, failed to recognize autologous viral variant sequences within cognate HLA class I-restricted epitopes. Consistent with selective perinatal HIV-1 transmission (61), a relatively homogeneous population of genetic variants was found in peripheral blood obtained from the infant after delivery, contrasting with the higher genetic diversity found in the peripheral blood obtained from the mothers. The predominant infant genotype often represented a minor maternal viral variant. Despite the presence of multiple immune escape variants in the transmitting mothers, the predominant viral strain in each of the three infants contained an epitope sequence well recognized by the maternal CTL response. One possible explanation for the presence of CTL-susceptible viral epitope variants in the infants is reversion of the transmitted escape variants to susceptible virus due to lack

of persistent immune pressure in the infants. Evidence against this hypothesis may be found in the evaluation of mother-infant pair 11113-11213. In this case, viral sequences susceptible to maternal CTL predominated in the infant despite the predominance of escape variants in the mother. Since infant 11213 did not share either of the HLA class I alleles that restricted transmitting mother 11113 CTL (Table 2), this infant would be unlikely to develop CTL that could maintain immune pressure through the epitopes recognized by those maternal CTL. This lack of immune pressure in the infant might be expected to allow for a reversion of the transmitted virus that carried CTL escape sequences to a more fit virus that carried CTL-susceptible epitope sequences. However, the phylogenetic analysis depicted in Fig. 3 clearly indicates that the virus transmitted from 11113 to 11213 was the CTL-susceptible form, despite the predominance of escape variants in the mother. These data add to a number of studies implicating escape from CTL recognition in the pathogenesis of HIV infection (4, 24, 48, 59, 60) but indicate that factors other than simple nonrecognition of the cognate epitope must be involved in perinatal transmission.

Selection for escape variants with mutations within well-defined HLA class I-restricted CTL epitopes can result in loss of reactivity of the epitope and act as a selective force that drives the evolution of the virus (4, 48, 49). This has been observed in acute infection when mutations within early targeted epitopes leads to loss of recognition (4, 49) and in chronic infection when mutations within CTL epitopes have been associated with more rapid disease progression (24). In addition, vastly different rates of disease progression found in a set of HLA-identical siblings infected by a common batch of contaminated factor VIII was associated with different patterns of amino acid changes in HLA class I-restricted epitopes and intensity of the CTL response (25). These studies and the present study indicate that immune selection pressure is exerted by CTL. Our study is unique in demonstrating that despite the presence of CTL escape variants in the mother, the virus that is established in the infant is a maternal CTL-susceptible form.

The increased frequency of CTL escape mutations in transmitting compared to nontransmitting mothers suggests a role for the CTL response in the transmission process, but the transmission of CTL-susceptible forms is counterintuitive. A number of factors could account for this. Viral variants can lead to antagonism of existing CTL responses (2, 36), but we were unable to find any evidence of this phenomenon (59a). Alternatively, the increased frequency of variants in the transmitting mothers may reflect an overall impairment of the ability to effectively contain viral replication. Critical additional CTL responses directed at type-specific epitopes present in vivo may be immune escape variants which went undetected because only laboratory strains of virus could be used for screening. Also, as these studies examined CTL and viruses derived from PBMC, it is possible that tissue-specific responses not represented in the peripheral blood are present in other relevant compartments (i.e., placenta or birth canal). Nevertheless, the data clearly demonstrate that variation within CTL epitopes is more prominent in women who transmit virus to their infants. The rapid and mutation-prone replication of the virus produces a diverse population that can effectively counter host-mediated selection pressures and perpetuate infection within the host (10). Whereas the viral sequences derived from the mothers show an accumulation of mutations in CTL epitopes consistent with positive selection for change, the infants have relatively little evidence of selective pressure for change. Thus, progressive shifts in the virus population in the HIV-1-infected mother, in contrast to the presumed relative evolutionary stasis in the infant, likely contribute to the observed disparity in amino acid sequences of the epitopes matched between an HIV-1-infected infant and the mother.

Ideally this study would have been performed with mothers matched for the same HLA antigens and target epitopes, but this was not feasible even in the large cohort of HIV-1-infected pregnant women we examined. The small sample size was a necessary constraint imposed by the detailed characterizations performed in this study, and the subjects included were limited to those in whom CTL clones could be established and maintained for full characterization. CTL responses were detected only if they were cross-reactive with the reference strains of virus used for the vaccinia constructs; thus, additional CTL responses may have been present but not detected. Furthermore, testing against reference strain rather than autologous virus might bias the analysis toward conserved epitopes in regions with structural and functional constraints that are less likely to participate in immune evasion. Nevertheless, these studies found greater amino acid changes within well-defined MHC class I-restricted epitopes for HIV-1-infected mothers who transmitted the virus to their infants relative to those mothers who did not. Thus, despite these experimental limitations, which are also inherent to other epitope mapping studies, the data clearly demonstrate the occurrence of CTL immune escape in the context of perinatal HIV-1 transmission. In summary, our data indicate that CTL immune escape occurs in perinatal HIV-1 transmission and provide further evidence of CTL-mediated immune selection pressure in this infection. Although antiviral drug intervention has been shown to decrease perinatal transmission (13, 56), our results suggest that interventions designed to augment cellular immunity to the viruses present in vivo, and minimizing immune escape, might also prove beneficial.

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