

Early HIV-1 Envelope-specific Cytotoxic T Lymphocyte Responses in Vertically Infected Infants

By Cheryl A. Pikora,* John L. Sullivan,* Dennis Panicali,[‡] and Katherine Luzuriaga*

From the *Department of Pediatrics, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605; and [‡]Therion Biologics Corporation, Cambridge, Massachusetts 02142

Summary

High frequencies of cytotoxic T lymphocyte precursors (CTLp) recognizing HIV-1 laboratory strain gene products have been detected in adults within weeks of primary infection. In contrast, HIV-1-specific CTLp are uncommonly detected in infants younger than 6 mo. To address the hypothesis that the use of target cells expressing laboratory strain *env* gene products might limit the detection of HIV-1 *env*-specific CTLp in early infancy, recombinant vaccinia vectors (vv) expressing HIV-1 *env* genes from early isolates of four vertically infected infants were generated. The frequencies of CTLp recognizing target cells infected with vv-expressing *env* gene products from early isolates and HIV-1 IIIB were serially measured using limiting dilution followed by *in vitro* stimulation with mAb to CD3. In one infant, the detection of early isolate *env*-specific CTLp preceded the detection of IIIB-specific CTLp. CTLp recognizing HIV-1 IIIB and infant isolate *env* were detected by 6 mo of age in two infants. In a fourth infant, HIV-1 IIIB *env* and early isolate *env*-specific CTLp were simultaneously detected at 12 mo of age. These results provide evidence that young infants can generate HIV-1-specific CTL responses and provide support for the concept of neonatal vaccination to prevent HIV-1 transmission. However, the early predominance of type-specific CTL detected in some young infants suggests that the use of vaccines based on laboratory strains of HIV-1 may not protect against vertical infection.

The vertical transmission of HIV-1 from an infected woman to her infant is the predominant mode of pediatric infection. Prospective studies estimate the rate of vertical transmission to be 14–40% (1). Vertical HIV-1 transmission can occur *in utero*, during delivery (intrapartum), or after birth through breastfeeding (1). In nonbreastfed populations, 45–70% of vertical infections occur in the intrapartum period. In developing countries, where breastfeeding is necessary and encouraged, up to 75% of infants may be infected at or after delivery.

In recent years, the incidence of vertical HIV-1 transmission has sharply increased (2) resulting in an urgent need to develop effective strategies to prevent vertical HIV-1 infection. Although the administration of zidovudine to mother–infant pairs has been shown to reduce significantly the risk of vertical HIV-1 transmission (3), the cost and intensity of the regimen render it impractical for use in developing nations, where most pediatric infections occur. Additionally, perinatal antiretroviral therapy would not prevent the vertical transmission of HIV-1 through breastfeeding beyond the neonatal period. A safe and effective active/passive vaccine regimen, begun at birth, therefore would be a more attractive strategy. Better understanding of the pathogenesis of vertical HIV-1 infection and the capability

of the young human infant to generate HIV-1-specific immune responses is crucial for the development of a vaccine to prevent vertical HIV-1 infection.

We and others have previously reported that HIV-1-specific cytotoxic T lymphocyte precursors (CTLp)¹ are uncommonly detected in early infancy (4, 5). Viral genotype/phenotype, early viral load, host genotype, timing of infection, and history of prior infections appear to be important factors in the generation of virus-specific CTL responses. Two lines of evidence suggested to us that the use of laboratory strain vaccinia vectors (vv) to detect HIV-1-specific CTL might underestimate the CTL repertoire in early infancy. First, analysis of vertically transmitted HIV-1 *env* sequences suggests that limited viral genotypes are transmitted or amplified after infection (6, 7). Second, Selin et al. (8) have reported higher frequencies of lymphocytic choriomeningitis (LCMV)-specific CTLp in animals who have experienced prior heterologous viral infections than in immunologically naive animals. Therefore, we hypothesized that type-specific responses might predominate in early infancy and that

¹Abbreviations used in this paper: CM, complete media; CTLp, cytotoxic T lymphocyte precursors; LCMV, lymphocytic choriomeningitis; m.o.i., multiplicity of infection; vv, vaccinia vectors.

the use of target cells expressing laboratory isolate gene products might limit the detection of HIV-1 env-specific CTLp in early infancy.

To address this hypothesis, HIV-1 *env* genes from early isolates of four vertically infected infants were PCR amplified, cloned, and used to generate recombinant vv. CTLp frequencies recognizing target cells infected with vv-expressing *env* gene products from early isolates and HIV-1 IIIB were serially measured from early to late infancy using limiting dilution followed by in vitro stimulation with mAb to CD3; split-well analysis allowed the evaluation of cross-reactivity of detected CTLp. In one infant, the detection of CTLp recognizing target cells expressing early isolate *env* preceded the detection of CTLp recognizing target cells expressing IIIB *env*. These type-specific CTLp detected in early infancy were later replaced by cross-reactive group-specific CTL. Cross-reactive *env*-specific CTLp recognizing HIV-1 IIIB and infant isolate *env* were detected by 6 mo of age in two infants. In a fourth infant, CTLp recognizing target cells infected with HIV-1 IIIB *env* and early isolate *env* were simultaneously detected at 12 mo of age. Implications for neonatal HIV-1 vaccine development are discussed.

Materials and Methods

Patients. Four infants with defined timing of infection and previously characterized CTL responses to HIV-1 IIIB *env* (4) were chosen for these studies. HIV-1 culture and DNA PCR

were positive in blood specimens obtained at birth and in all subsequent specimens from two infants (VI-05 and VI-06), suggesting in utero infection (9). HIV-1 culture and DNA PCR were negative on specimens obtained from two other infants (VI-08 and VI-11) at birth but were positive by 1 mo of age, suggesting late in utero or intrapartum infection. None of the infants were on antiretroviral therapy at the time that isolates were obtained for use in the preparation of vv constructs.

HIV-1 IIIB *env*-specific CTLp were previously detected in blood samples obtained during early infancy from only 1 (VI-06) of the 4 infants (4). HIV-1 CTLp were detected in cord blood and subsequent specimens from VI-06. HIV-1 gag-specific CTLp were detected as early as 3 mo of age in VI-11; *env*-specific CTLp were not detected through 12 mo of age. In VI-05 and VI-08, HIV-1 *env*-specific CTLp were not detected until 10–11 mo of age. Human Studies Committee approval and individual informed consent from the guardian of each infant were obtained before we conducted these studies. Table 1 summarizes sequential measures of peripheral blood HIV-1 load and CD4 counts of each infant studied as well as the ages at which *env* genes were amplified from infant viral isolates for cloning and insertion into vv.

Lymphocyte Separation and Cryopreservation. PBMC were isolated from freshly drawn heparinized blood by Ficoll–Paque (Pharmacia, Piscataway, NJ) density centrifugation (10). PBMC were viably cryopreserved using a KRYO 10 Series cell freezer and stored in liquid nitrogen until use.

Preparation of Genomic DNA. PBMC cultures were performed according to the AIDS Clinical Trials Group virology consensus protocols (11). Supernatants from these cultures were used to establish low (1–2) passage viral cultures at the timepoints specified in Table 1. In brief, 10⁷ HIV-1 seronegative donor PHA blasts

Table 1. Sequential Measures of Peripheral Blood HIV-1 Load and CD4 Counts of Infants Studied

Patient	Age	DNA PCR	Plasma culture*	PBMC culture†	RNA§	%CD4	Absolute CD4
VI-05	Birth	Pos	0	0	ND	50	ND
	1 mo	ND	1,562	625	2,533,526		
	2 mo	ND	62	625	297,670	20	3,015
	6 mo	ND	ND	ND	ND	14	1,230
VI-06	Birth	Pos	0	25	49,000	ND	ND
	1.5 mo	Pos	12.5	25	210,000	15	1,509
	3 mo	ND	62.5	125	340,000	19	1,369
	5 mo	ND	ND	ND	1,300,000	25	1,110
VI-08	Birth	Neg	ND	0	ND	41	1,051
	20 d	Pos	63	125	ND	50	2,090
	1.5 mo	ND	ND	ND	685,169	ND	ND
	5 mo	Pos	0	1	321,712	36	2,547
VI-11	6 mo	ND	ND	ND	57,302	ND	ND
	Birth	Neg	0	0	1,353	35	1,105
	2 mo	Pos	ND	0	47,654	31	2,371
	3 mo	ND	0	0	ND	34	ND
	7 mo	ND	0	5	21,555	29	2,354

The ages at which *env* genes were amplified from infant viral isolates for cloning and insertion into vv are denoted in bold type.

*TCID₅₀/ml plasma.

†TCID₅₀/10⁶ PBMC.

§RNA copies/ml plasma.

cultured in complete media (CM) containing RPMI, 10% FCS, 50 µg/ml gentamicin, and 5 U/ml IL-2 were pelleted, resuspended in 1 ml of viral culture supernatant, and incubated for 1 h at 37°C in 5% ambient CO₂. The cells were then washed and resuspended in CM; the p24 antigen content of the supernatant was monitored daily by enzyme immunoassay. On day 5 of culture, high molecular weight DNA was purified by a standard proteinase K digestion-isopropanol precipitation technique.

PCR Primers and Conditions. Oligonucleotide primer sequences were chosen from regions directly upstream and downstream of the initiation and termination codons of the *env* gene, respectively. The following two primers were used: MNA, 5'-GCG-AAAGAGCAGAAGACAGTGGC-3' (corresponding to positions 6197-6220 of the NL4-3 genome) and MN13, 5'-CAGCTC-GTCTCATTCTTTCCC-3' (positions 8836-8857). PCR mixtures consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM each of the four deoxynucleoside triphosphates, 2.5 mM MgCl₂, 10 pmol of each primer, 200 ng of DNA, and 2.5 U of AmpliTaq polymerase (Perkin-Elmer, Foster City, CA), which was added during an initial 3-min denaturation step. Amplification was conducted for 30 cycles in a thermal cycler (Perkin-Elmer). Each cycle consisted of three steps: denaturation (94°C for 60 s), primer annealing (60°C for 90 s), and extension (72°C for 3 min), with a final extension at 72°C for 10 min.

Heteroduplex Formation and Sequence Diversity Determination. The V1 to V3 region of *env* was PCR amplified from the cloned PCR *env* products using primers 209 (positions 6453-6470) and 218 (positions 7382-7399). PCR conditions were identical to those described above except for a MgCl₂ concentration of 4 mM, an

annealing temperature of 55°C, and the absence of a hot start. After the internal labeling of PCR products with [³²P]dCTP, heteroduplexes were formed between labeled and unlabeled products and DNA fragments were separated in a neutral 5% polyacrylamide gel (12). DNA fragments were separated using the Model S2 Sequencing Gel Electrophoresis Apparatus (GIBCO BRL, Gaithersburg, MD) at 30 mA for 12 h. Sequence comparisons and diversity estimates were determined using best-fit analysis in GCG using the Genetics Computer Group's Wisconsin Sequence Analysis Package version 8.0.1 with the use of the default program parameters.

Molecular Cloning and Recombinant *vv* Generation. The entire 2.6-kb coding region of *env* was PCR amplified from proviral DNA at each of two early timepoints (first and 3-6-mo isolates) from each patient; the PCR product was gel purified with Eluquick (Schleicher & Schuell, Keene, NH) and ligated to the plasmid vector pCR3 (InVitrogen, San Diego, CA). After ligation, the reaction mixture was introduced into TOP10F' cells by CaCl₂ transformation. Cells were plated on LB-carbenicillin agar plates and incubated overnight at 37°C; selected colonies were then expanded in 3-ml cultures containing LB-ampicillin overnight at 37°C. Clones with inserts of appropriate size, as judged by restriction enzyme digestion, were sequenced at the SP6 or T7 promoter sites within pCR3 adjacent to the insert to determine orientation using the Sequenase Version 2.0 sequencing kit (United States Biochemicals, Cleveland, OH). Clones were then grouped according to digestion patterns obtained with StuI, AvaI, BamHI, KpnI BglI, and HindIII and a predominant clone was selected from each timepoint for subcloning (Table 2).

Table 2. Grouping of Patient Isolate *env* Clones within pCR3 Based upon Restriction Digests and Choice of Predominant Clones for Preparation of *vv*

Patient	Age	Group	Env clones*	Recombinant <i>vv</i>
VI-05	2 mo	05-2M-A	3-2, 3-4 , 3-5, 3-7, 3-8, 3-10	vvVI-05-2M
		05-2M-B	3-3	
VI-06	6 mo	05-6M-A	4-2 , 4-4, 4-5, 4-6, 4-8, 4-9	vvVI-05-6M
		06-1D-A	1-2, 1-3	
VI-06	Birth (1 d)	06-1D-B	1-9	
		06-1D-C	1-11, 1-12, 1-13 , 1-15, 1-20	vvVI-06-ID
		06-1D-D	1-18	
		06-6M-A	2-1, 2-2, 2-3, 2-4, 2-7, 2-8, 2-9, 2-10	‡
VI-08	20 d	06-6M-B	2-5	‡
		08-20D-A	5-4	
VI-08	6 mo	08-20D-B	5-6	
		08-20D-C	5-2 , 5-3, 5-8, 5-9, 5-10	vvVI-08-20D
		08-6M-A	6-1	
VI-11	1 mo	08-6M-B	6-2 , 6-3, 6-4, 6-7, 6-8, 6-9, 6-10	vvVI-08-6M
		11-1M-A	7-1	
VI-11	3 mo	11-1M-B	7-2, 7-5, 7-7, 7-10, 7-11 , 7-16, 7-19	vvVI-11-1M
		11-1M-C	7-12	
		11-3M-A	8-3	
		11-3M-B	8-4 , 8-11, 8-14, 8-16	vvVI-11-3M

* *env* genes amplified by PCR and ligated into pCR3 (InVitrogen); clones chosen for preparation of *vv* are in bold type.

‡ *vv* not made from this time point due to instability of the *env* insert.

The predominant clone from each timepoint was digested with BamHI and XhoI and ligated into the BamHI-XhoI site of pAbT4587A (created by the addition of an XhoI site within the pAbT4587 vector provided by Therion Biologics, Cambridge, MA). After ligation, the reaction mixture was introduced into DH5a cells (Therion Biologics, Cambridge, MA) by CaCl₂ transformation. Cells were grown as described above and clones were again screened by restriction enzyme digests.

Recombinant vv expressing infant isolate *env* gene products were generated, amplified, and titered according to the methods of Mazzara et al. (13). Each *env*-recombinant vv expressed gp160 and its cleavage products as determined by radio immunoprecipitation. In addition, each of these vv was able to sensitize target cells to antibody-dependent cell-mediated cytotoxicity (ADCC) lysis (Pugatch, D., K. Luzuriaga, and J.L. Sullivan, manuscript submitted for publication).

Limiting Dilution Assays of CTL Precursors. HIV-1 *env*-specific CTLp frequencies were estimated using previously described methods (4, 14). To minimize potential variability in assay conditions and to allow comparison of results between the timepoints studied for each infant, all limiting dilution cultures for each infant were set up and all CTLp assays were performed at the same time and with the same reagents. Cryopreserved PBMC were thawed and diluted at 16,000 to 250 lymphocytes per well in 24 replicate wells of 96-well microtiter plates; 2.5–5.0 × 10⁴ irradiated PHA blasts from HIV-1-uninfected donors and mAb to CD3 (12 F6; 0.1 μg/ml; provided by Dr. J.T. Wong, Massachusetts General Hospital, Boston, MA) were added to each well and the plates were incubated at 37°C in R10 with 30 U/ml IL-2 for 7–10 d. Wells were then split and assayed for cytotoxicity on ⁵¹Cr-labeled autologous B lymphoblastoid cell lines infected either with vac alone or with vv-expressing IIIB or patient isolate *env* proteins (M.O.I. = 5:1). The fraction of nonresponding wells was defined as the number of wells in which lysis did not exceed 10% (VI-11) or 20% (VI-05, VI-06, VI-08). Percentage of lysis was calculated for each well using the formula 100 × (test cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm); the CTLp frequency and 95% confidence limits were calculated using the maximum likelihood method (15) and a spreadsheet provided by Dr. S. Kalams (Massachusetts General Hospital, Boston, MA). Split well analysis was used to examine the cross-reactivity of CTLp.

Quantitation of Plasma HIV-1 RNA. Plasma HIV-1 RNA levels were measured after reverse transcription and PCR amplification using a commercial assay (Roche Diagnostic Systems, Branchburg, NJ), as directed by the manufacturer.

Results

HIV-1 Infant Isolate *env* Sequences Are Homogeneous and between 8–10% Divergent from IIIB *env*. We began by examining the degree of diversity between infant isolate *env* and IIIB *env*. Examination of sequences through the V3 loops suggested relative homogeneity of first isolates (data not shown). Divergence in nucleotide sequence between HIV-1 IIIB and individual infant isolate *env* was then estimated using heteroduplex tracking assays and sequencing. Fig. 1 demonstrates that heteroduplexes formed by combining individual infant isolate and IIIB *env* migrate at rates between those of heteroduplexes formed by combining IIIB and MN *env* or IIIB and p08-6-20D (an *env* clone derived from the virus of patient VI-08). Sequence com-

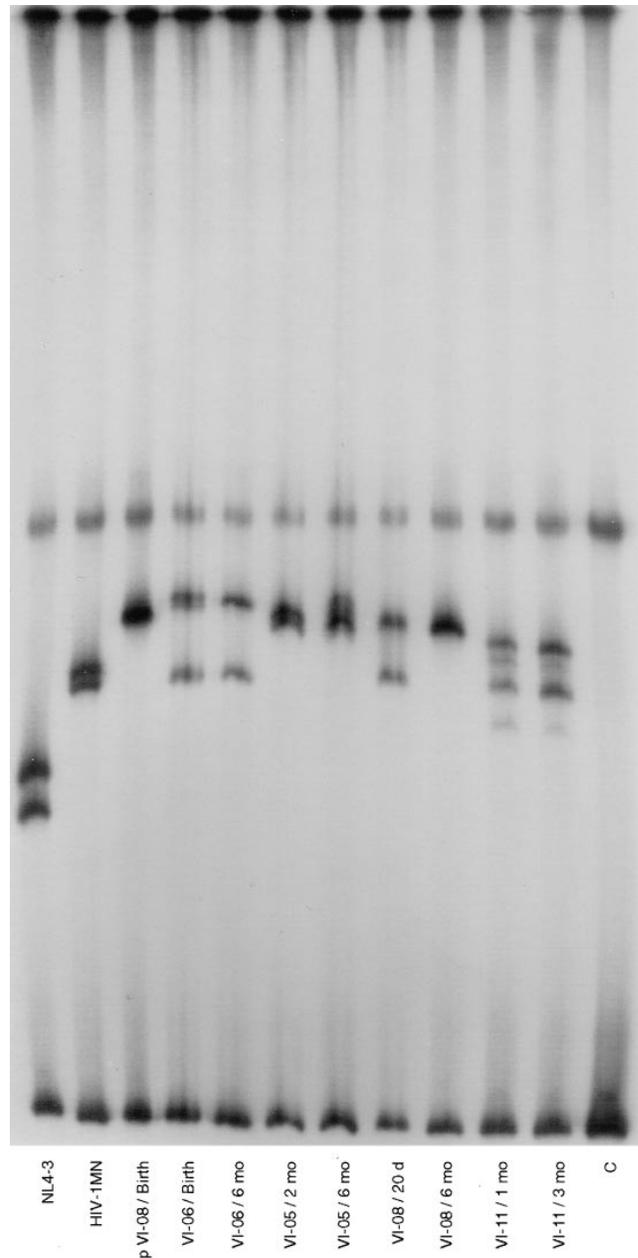


Figure 1. Heteroduplex analysis of homology between patient isolate DNA and IIIB *env* sequences. *Env* fragments (946 bp in the VI-1-V3 regions) were PCR amplified from genomic DNA extracted from PBMC infected with patient HIV-1 isolates or from a plasmid containing BH10 (IIIB) *env* (pAbT4603). The PCR product of pAbT4603 was internally radiolabeled during the PCR and combined with the PCR products of the proviral templates of the patient to form heteroduplexes as described in Materials and Methods. Lanes 1–3 are clones of known sequence probed with pAbT4603. Lane 1 is a PCR product of the NL 4-3 strain of HIV-1, lane 2 is a PCR product of the MN strain of HIV-1 (prototypic clade B strain of HIV-1), and lane 3 is a PCR product from an *env* clone derived from patient VI-08 at 1 d of age (pJA-6-1D). Lanes 4–11 are PCR products amplified from patient proviral DNA (VI-06-1D and VI-06; VI-05; VI-05-6M; VI-08-20D and VI-08-6M; VI-11-1M and VI-11-3M; Table 2). Lane 12 is a negative control of probe alone.

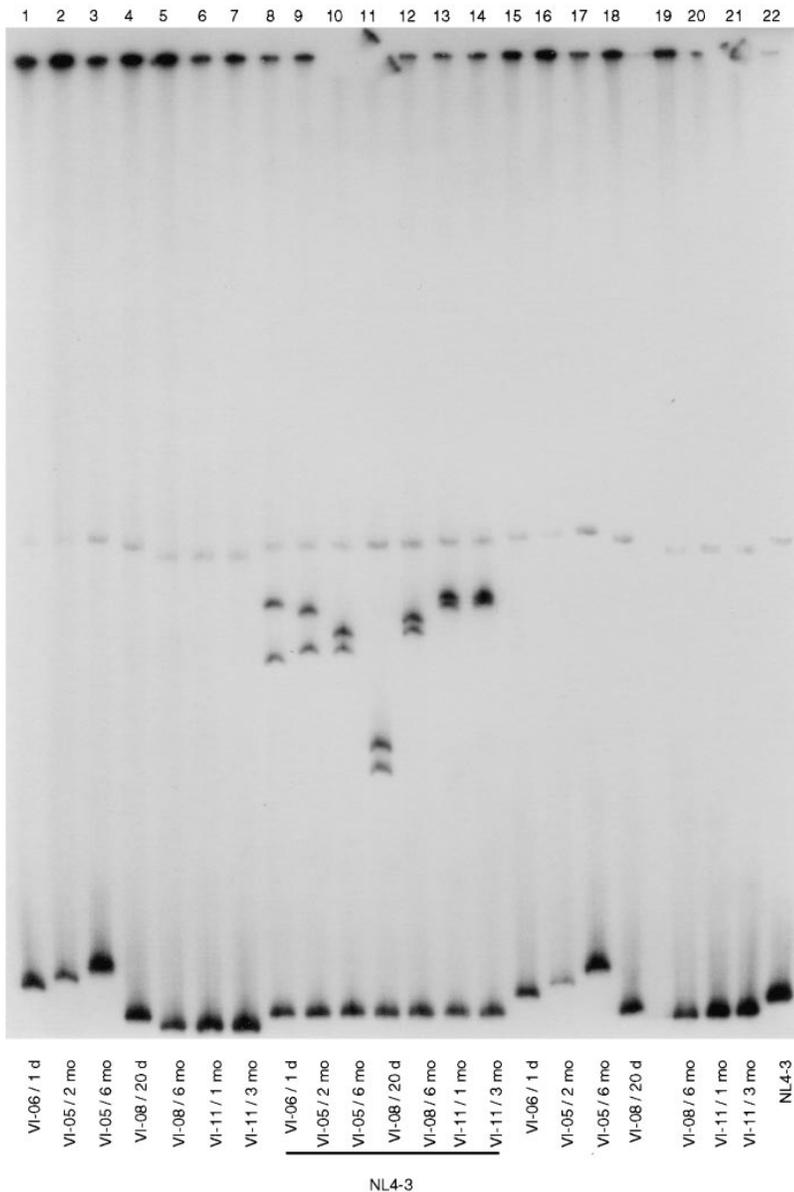


Figure 2. Heteroduplex analysis of cloned and sub-cloned *env* genes. *env* fragments (946 bp in the V1–V3 regions) were PCR amplified from *env* clones within the pCR3 and pAbT4587A backbones and from pNL4-3. The PCR product of *env* clones within pCR3 and pNL4-3 were internally radiolabeled during the PCR. The PCR products of *env* clones within pCR3 were combined with the PCR products of the identical *env* subclones within pAbT4587A to form heteroduplexes as described in Materials and Methods. The radiolabeled PCR product of pNL4-3 was also combined with PCR products of the *env* clones within pAbT4587A to form heteroduplexes. Lanes 1–7 are subcloned *env* genes probed with identical genes ligated into pCR3. Lanes 8–14 are subcloned patient isolate *env* genes probed with pNL4-3. Lanes 15–22 are negative controls containing radiolabeled PCR products alone.

comparisons of a 946-bp region spanning V1 to V3 indicate that IIIB and MN *env* differ from each other by 8.3%, whereas IIIB and p08-6-20D differ by ~10%. Therefore, the degree of heterogeneity between individual infant isolate and IIIB *env* sequences is between 8–10%.

The Generation of Recombinant *vv* from Early Infant Viral Isolates. The entire 2.6-kb coding region of *env* was amplified from proviral DNA at two early timepoints (and first 3–6-mo isolates) from each infant, ligated into pCR3 (Invitrogen), and digested with *Stu*I, *Ava*I, *Bam*HI, *Kpn*I, *Bgl*II, and *Hind*III. One to four distinct patterns of restriction digests were obtained from each set of clones derived from virus from each patient at a single timepoint and were designated as groups A, B, C, and D. The group containing the largest number of clones was considered to be the predominant group and one clone was then chosen from each

predominant group for subcloning (Table 2). The instability of the *env* gene from the viral isolate of patient VI-06 at 6 mo of age in pAbT4587A prevented its subcloning. Therefore, only one *env* subclone, from 1 d of age, was derived from virus of infant VI-06.

The gel isolation of multiple *env* genes for subcloning could potentially result in contamination of one *env* gene with another. Heteroduplex analysis was employed to ascertain whether the subcloned *env*s were identical to the original clone from which they were derived. Absence of contaminating *env* sequences was shown by the lack of heteroduplex formation between cloned *env* fragments and their identical subclone (Fig. 2). In addition, *env* coding sequences from pNL4-3, a molecular clone used frequently in our laboratory, were also used to form heteroduplexes with the *env* clones derived from infant viral isolates to en-

Table 3. *HIV-1 env-specific CTLp Frequencies Over Time*

Patient	vv*	Cord blood	CTLp (95% confidence intervals)					
			2 mo	4 mo	6 mo	7 mo	12 mo	19 mo
VI-06	vac	34 (23–50)			0.5 (0.03–8)			
	IIIB	13 (7–23)			153 (118–200)			
	vvVI-06-1D	64 (47–87)			197 (151–257)			
VI-05	vac	3 (1–10)					65 (48–89)	
	IIIB	3 (1–9)					219 (169–284)	
	vvVI-05-2M	8 (4–17)					129 (99–168)	
	vvVI-05-6M	ND					55 (40–76)	
VI-08	vac			88 (66–116)	50 (36–70)			19 (12–32)
	IIIB			240 (184–311)	50 (35–70)			201 (154–262)
	vvVI-08-20D			165 (127–213)	130 (99–170)			81 (60–108)
	vvVI-08-6M			202 (156–261)	80 (60–107)			175 (135–229)
VI-11	vac		8 (4–17)			3 (1–10)	95 (72–125)	
	IIIB		23 (14–36)			4 (1–11)	286 (219–373)	
	vvVI-11-1M		136 (105–177)			42 (29–60)	537 (403–717)	
	vvVI-11-3M		138 (107–180)			70 (52–94)	233 (180–303)	

*vac, vaccinia alone; IIIB, vv expressing IIIB env gp160. See Table 2 for explanation of vaccinia vectors expressing infant isolates.

sure that none contained the pNL4-3 env sequence. The formation of heteroduplexes of varying mobility between pNL4-3 and the cloned env fragments further verified lack of contamination and demonstrated that each of the env clones was unique.

HIV-1 env-specific CTL Responses in Early Infancy. Beginning in early infancy, CTLp frequencies were measured sequentially in the peripheral blood of the four infants studied. In three infants, env-specific CTLp were detected by 6 mo of age (Table 3).

HIV-1-specific CTLp recognizing autologous env gene products were detected in the peripheral blood of a second infant (VI-11) at 3, 7, and 12 mo of age and preceded the first detection of CTLp recognizing HIV-1 IIIB env gene products at 12 mo of age (Table 3). HIV-1-specific CTLp recognizing first (20-d) isolate and IIIB env gene products were detected by 4 mo in another intrapartum-infected infant (VI-08). In infant VI-06, HIV-1-specific CTLp recognizing first isolate and IIIB env gene products were detected by 6 mo of age. HIV-1-specific CTLp were not detected in the cord blood of infant VI-05, but CTLp recognizing IIIB and infant isolate env gene products were detected at 12 mo of age.

Evaluation of Cross-reactivity of HIV-1 env-specific CTLp Using Split-well Analysis. Genotypic analysis of sexually (16) and vertically (6, 7) transmitted HIV-1 strains suggests that limited viral genotypes appear to be transmitted or amplified after infection. After infection, diversification of viral species may occur through reverse transcriptase error and various selective pressures (for review see reference 17). New populations of CTL may expand in an infected indi-

vidual in response to evolving quasispecies of HIV-1, resulting in a broadening of the HIV-1 immune response. Split-well analysis allowed us to examine CTLp cross-reactivity on a clonal level. Fig. 3 illustrates CTLp cross-reactivity over time.

HIV-1 env-specific CTLp detected in VI-11 in early infancy exclusively recognized early infant isolate env, whereas HIV-1-specific CTLp detected in later infancy were primarily cross-reactive. In VI-06 and VI-08, cross-reactive CTLp recognizing early infant isolate and IIIB env were detected as early as 6 and 4 mo of age, respectively. At 6 mo of age, only CTLp recognizing target cells infected with vv expressing the 20-d isolate were detected in VI-08, while cross-reactive CTLp were detected again at 19 mo. Since virus-independent in vitro stimulation was used to expand CTLp, the CTLp frequencies detected are likely reflective of the CTLp frequencies in vivo. However, at the relatively low CTLp frequencies detected at 6 mo, sampling error might explain the sole detection of type-specific CTLp.

HIV-1-specific CTLp Activity Detected in Early Infancy Is CD8 T Cell Mediated. HIV-1 env-specific cytolysis may be CD8 T cell mediated and HLA class I-restricted or NK cell-mediated cytolysis through ADCC (18). Unfortunately, the limited PBMC repository available from early infancy precluded the use of CD4, CD8, or NK-depleted PBMC populations in the limiting dilution assays. To determine the phenotype of the effector cells in the limiting dilution assays, we performed limiting dilution assays in which a bispecific (anti-CD3, CD8) mAb was used instead of mAb to CD3 (19). Use of this antibody led to the depletion of CD8 T cells from the LDA wells (<1% CD8 T cells compared with 45% CD8 T cells in the wells treated with

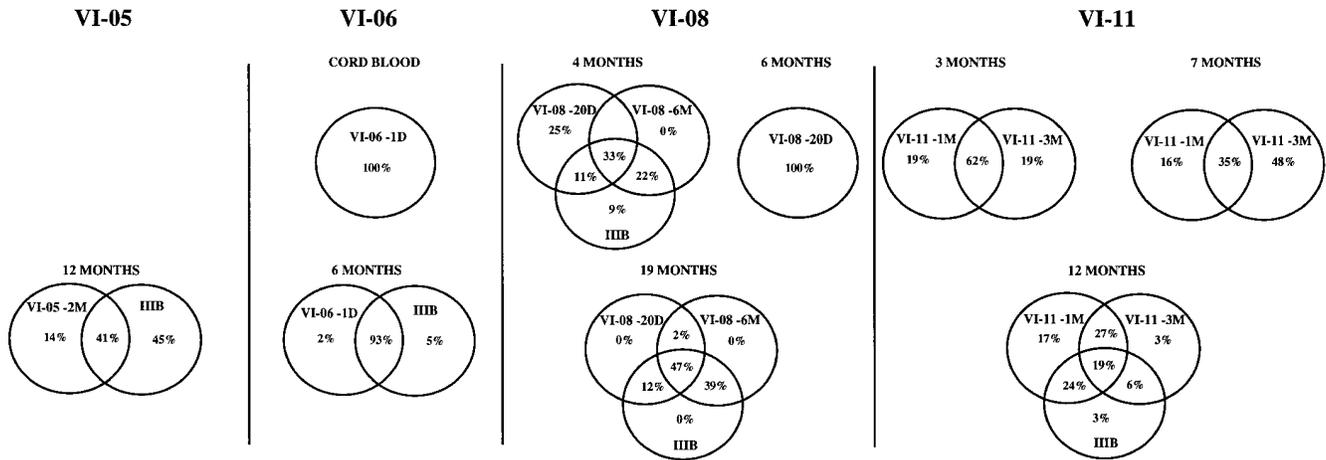


Figure 3. Evaluation of env-specific CTLp cross-reactivity using split-well analysis. Proportional CTLp frequencies at each designated time are summarized as Venn diagrams. See Tables 1 and 2 for descriptions of patients and vv-expressing infant isolates.

mAb to CD3 alone) and a 55–90% reduction in env-specific CTLp frequency compared with wells treated with mAb to CD3 alone. These studies suggest that the env-specific CTLp detected in the assays were mediated by CD8 T cells.

Detection of HIV-1 Env-specific CTLp in Early Vertical Infection, Viral Load, and Clinical Disease Progression. In three infants, HIV-1-specific CTL recognizing early infant isolate gene products were detected before 6 mo of age. Infant VI-11, in whom HIV-1 gag- and early isolate env-specific CTL were detected by 3 mo of age, has remained only mildly symptomatic without evidence of immune suppression (CDC A1; reference 20) at 3 yr of age. However, as previously described (4), infant VI-06 experienced a rapid increase in viral load and a rapid decline in peripheral blood CD4 T cell numbers after birth despite the detection of HIV-1 gag- and env-specific CTL in cord blood and 3-wk specimens. While env-specific CTLp were detected in infant VI-08 by 4 mo of age, this infant developed HIV-1 encephalopathy and severe CD4 depletion (CDC C3) by 3 yr of age. The early detection of HIV-1 env-specific CTLp in the latter two infants suggest that HIV-1-specific CTL alone may not protect against CD4 depletion or disease progression.

Discussion

In assays using target cells expressing laboratory strain HIV-1 gene products, HIV-1-specific CTLp have been uncommonly detected in early infancy. This study addressed the hypothesis that type-specific responses might predominate in early infancy and that the use of target cells expressing laboratory isolate gene products might limit the detection of HIV-1-specific CTL in early infancy. To address this hypothesis, HIV-1 *env* genes from early isolates of four vertically infected infants were PCR amplified, cloned, and used to generate recombinant vv. The frequencies of CTLp recognizing target cells infected with vv-expressing *env* gene products from early isolates and HIV-1 IIB were serially measured from early to late infancy using limiting di-

lution followed by in vitro stimulation with mAb to CD3. HIV-1-specific CTLp were detected before 6 mo of age in three infants. In one infant, the detection of CTLp recognizing target cells expressing early isolate *env* preceded the detection of CTLp recognizing target cells expressing IIB *env*; these type-specific CTLp detected in early infancy were later replaced by cross-reactive group-specific CTL. In two other infants, early group-specific responses were detected. In a fourth infant, CTLp recognizing target cells infected with HIV-1 IIB *env* and early isolate *env* were simultaneously detected at 12 mo of age. These results reconfirm that young infants can generate HIV-1-specific CTL responses and provide support for the concept of perinatal vaccination to prevent HIV-1 transmission.

The generation of vv-expressing infecting strain gene products was central to our studies. To minimize the chance of amplifying defective HIV-1 *env* sequences, we chose to use *env* genes amplified from cultured virus to construct the env-expressing recombinant vv. Whereas several studies have demonstrated the outgrowth of variant strains of limited diversity in viral coculture that did not predominate in vivo at the timepoint at which the culture was established (21, 22), several lines of evidence suggest that the isolates used were representative of the in vivo population of viruses. First, studies that have compared the selection of variants in culture to those present in vivo have maintained these viral cultures up to at least 28 d. The *env* genes used to generate recombinant vv in our studies were amplified from minimally passaged virus cultured for less than 1 wk. Second, the absence of anti-retroviral therapy in these patients at each of the timepoints from which the env vaccinia recombinants were generated supports the notion that these variants were not suppressed in vivo and expanded in vitro in the absence of drug. Third, the ability of CTL precursors from these patients to recognize env protein products from the env sequences derived from cultured virus suggests that these sequences were present in vivo.

Minimal diversity in *env* sequences of HIV-1 strains isolated from adults and infants early in primary infection has

been reported (6, 7, 16). Although HIV-1-specific CTL expand in response to a homogeneous population of variants in both populations of infected individuals, group-specific CTL appear to be detected more commonly in adult primary infection than in vertical primary infection (23, 24). Therefore, the type specificity of early infant CTL responses is not likely due to a homogeneous starting population of virus.

Adults possess memory CTL that may cross-react with HIV-1 proteins and expand in response to HIV-1 infection in concert with antigen-naïve CTL precursors. CTL expansion upon acute HIV-1 infection in adults may be similar to that observed by Selin et al. (8) in mice. They observed that acute Pichinde virus infection in LCMV immune mice resulted in expansion of CTL that were cross-reactive with both LCMV and Pichinde virus. Cross-reactive memory CTL originally expanded in response to a previous non-HIV-1 antigenic encounter may possess TCRs with lower affinity for HIV-1 antigen. A lower affinity interaction may allow for a greater degree of promiscuity in recognition of epitopes of HIV-1 gene products on various HIV-1 strains. The group-specific CTL responses detected in infected adults during or shortly after seroconversion may be due in part to the contribution of this expanded non-HIV-1-specific memory pool of CTL.

In contrast, HIV-1 infection occurs in infants whose immune systems have not been primed by previous antigenic exposure. Therefore, potentially cross-reactive memory CTL may not exist. Diversification of the viral variant population over time may eventually result in the expansion of CTLp populations recognizing a greater number of viral epitopes with the concomitant ability to recognize these epitopes on more than one strain of HIV-1.

Alternatively, type-specific responses detected in some young HIV-1 vertically infected infants may be due to partial tolerance. Vertical HIV-1 infection occurs at a time when the cellular immune system is being vigorously edited on the basis of its ability to discriminate between self and nonself. HIV-1 antigen may be viewed as self-antigen during this process and HIV-1-reactive CTL may be deleted or anergized. The ability of HIV-1 to infect and replicate within professional antigen presenting cells such as macrophages and its presence on dendritic cells may allow for activation of a small population of HIV-1-reactive CTL that escape tolerance induction. This partial break in tolerance may originally be directed at a limited array of viral epitopes, resulting in a type-specific CTL response. Recognition of these initial epitopes by CTL may lead to an expansion of CTL responsiveness in which a progressively greater number of viral epitopes may be recognized. Expansion in epitope recognition may subsequently result from the phenomenon of epitope spreading (25) in concert with a diversification of HIV-1 variants toward viral gene products whose sequences bear less resemblance to the original tolerizing variants. The group-specific CTL responses detected by 12 mo of age may evolve in this manner.

In summary, our studies indicate that young infants are capable of generating virus-specific CTL in response to viral infection and some support the development of a neonatal HIV-1 vaccine. However, the detection of type-specific immunity in some young infants suggests that a vaccine based upon laboratory strains of HIV-1 may not be protective. For optimal efficacy, it will likely be necessary to use gene products from viral strains isolated from patients in targeted geographical regions as immunogens.

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Address correspondence to K. Luzuriaga, M.D., Department of Pediatrics, Program in Molecular Medicine, University of Massachusetts Medical School, BioTech II, Suite 318, 373 Plantation Street, Worcester, Massachusetts 01605.

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