A Dissertation Presented

By

Cheryl A. Pikora

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DECEMBER 15, 1995

IMMUNOLOGY/VIROLOGY

TYPE-SPECIFIC IMMUNITY IN HIV-1 VERTICALLY INFECTED INFANTS

A Dissertation Presented

By

Cheryl A. Pikora

Approved as to style and content by:

Raymond Welsh, Ph.D., Chair of Committee

Harriet Robinson, Ph.D., Member of Committee

Francis Ennis, M.D., Member of Committee

Gregory Viglianti, Ph.D., Member of Committee

Mario Stevenson, Ph.D., Member of Committee

John L. Sullivan, M.D., Dissertation Mentor

Thomas Miller, Ph.D., Dean of the Graduate School of Biomedical Sciences

Immunology/Virology Program

December 15, 1995

# DEDICATION AND ACKNOWLEDGEMENTS

I dedicate this thesis to my mother. She has shared the frustrations as well as the joys of this research with me and has patiently listened and given me encouragement and support every step of the way. I also dedicate this thesis to the memory of my father. He was always proud of my achievements and made me believe that I could be anything I chose to be. Both my mother and father believed in the value of education and labored hard all their lives to ensure that I would have every opportunity to achieve my goals. So it seems only fitting that this thesis, representing a milestone of educational achievement in science, should be dedicated to my parents.

I also would like to acknowledge my mentor, Dr. John L. Sullivan. Through all the years that I have known him, he has served as a role model for me as an enthusiastic, talented and dedicated scientist and clinician. I would like to thank him for believing in me and encouraging me to pursue my goals. He has been instrumental in my growth not only as a scientist and medical student, but also as a person. He has been an advisor to me in the truest sense of the word: by taking a personal interest in my endeavors, giving me advice and providing me with opportunities to further my knowledge.

I would like to thank Dr. Gregory Viglianti who was always willing to make time to help me. His advice and assistance and his willingness to listen were invaluable to the completion of my project. As a rotation student in his lab before beginning this project, he provided me with a base from which I was able to build in performing many of the molecular biology techniques necessary for my thesis project. He continued to broaden this base with his input and suggestions during the execution of my thesis research. In addition I would also like to thank him for his friendship and support which were equally as invaluable.

I would like to thank my close friend Philip Morway who has been a part of my life for many years and has never let me down when I needed advice or a listening ear. He has shared in my happiness and frustration throughout the years of this endeavor and has always been a source of strength and support.

Finally, I would also like to acknowledge all of the people in the Sullivan laboratory who kept my spirits up and added a great deal of enjoyment to my stay there. The long hours necessary to complete my research were more easily borne with the lively and warm companionship provided by this group of people.

# ABSTRACT

High frequencies of CTL recognizing laboratory strains of HIV-1 are present in HIV-1 infected adults as early as preseroconversion. The presence of HIV-1 specific CTL during primary infection has been correlated with better control of early viremia and a more delayed onset of CD4 lymphocyte loss. Previous experiments in our laboratory have demonstrated that, unlike HIV-1 infected adults, the majority of vertically infected infants lack CTL which recognize laboratory strains of HIV-1 within the first year of life. ADCC antibody responses against laboratory strains of HIV-1 env gene products are also delayed until at least two years of age. As a possible correlate, disease progression is also more rapid in vertically infected infants.

We hypothesized that HIV-1-specific CTL are type-specific in early infancy and that the use of target cells expressing laboratory strain gene products might limit the detection of HIV-1-specific CTL. To address this hypothesis, HIV-1 env genes from early isolates of four infants were PCR amplified, cloned, and used to generate recombinant vaccinia vectors (vv). The frequencies of CTL precursors (CTLp) recognizing env gene products from autologous isolates and the IIIB strain of HIV-1 were measured at time points from early infancy to 19 months using limiting dilution analysis (LDA). ADCC titers were also measured against autologous and IIIB env gene products at 4 time points spanning 2 months to 2 years of age.

CTL precursors from 3 of 4 of these patients were specific only for autologous HIV-1 env gene products during the first 6 to 12 months of age. A pattern of CTL responsiveness was observed in these 3 patients in which type-specific CTL precursors observed in early infancy were replaced by cross-reactive, group-specific CTL by 6 to 12 months of age. CTL precursors from a fourth patient at 12 months of age recognized IIIB env and 1 out of 2 envs derived from 2 autologous viral isolates.

High titers titers of ADCC antibodies against autologous env were detected in two infants prior to the detection of ADCC antibodies to IIIB. In two other infants, group specific ADCC antibody responses were detected in late infancy.

Our results demonstrate that young infants can mount HIV-1 specific CTL and ADCC responses. The ability of young infants to mount cellular immune responses to HIV-1 also provides support for the concept of perinatal vaccination to prevent HIV-1 transmission. Furthermore, the lack of broadly-reactive CTL in early infancy suggests that the use of vaccines based on laboratory strains of HIV-1 may not afford protection from vertical infection.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

## ABBREVIATIONS

| | |
|---|---|
| ADCC | Antibody dependent cellular cytotoxicity |
| AIDS | Acquired immunodeficiency syndrom |
| B-LCL | B lymphoblastoid cell line |
| CD | Cluster determination |
| ConA | Concanavalin A |
| CTL | Cytotoxic T lymphocyte |
| DMSO | dimethylsulfoxide |
| EBV | Epstein-Barr virus |
| FCS | Fetal calf serum |
| HIV-1 | Human immunodeficiency virus |
| HSV | Herpes Simplex virus |
| hu-PBMC-SCID | human peripheral blood mononuclear cell-reconsituted SCID mouse |
| IL-2 | Interleukin-2 |
| LCMV | Lymphocytic choriomeningitis virus |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| NK | Natural killer |
| NP | Nucleoprotein |
| PBL | Peripheral blood leukocyte |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pCTL | Cytotoxic T lymphocyte precursor |

| | |
|---|---|
| PHA | Phytohaemmaglutinin |
| PWM | Poke weed mitogen |
| RIPA | Radio-immunoprecipitation |
| SCID | Severe combined immunodeficiency |
| vv | Vaccinia virus |

# CHAPTER 1. INTRODUCTION

## A. General Characteristics of HIV-1 Infection

Since 1983, when the first strain of HIV-1 was identified and sequenced by Gallo and Montagnier, a multitude of strains of HIV-1 have been isolated and sequenced from infected individuals and categorized on the basis of their degree of nucleotide identity. From this data it was observed that individual HIV-1 strains taken from a particular geographic location were closely related and therefore were organized into "clades" based upon this similarity. As a result, particular HIV-1 laboratory strains have been considered to be representative of the clade as a whole and used to study HIV-1-specific immune responses within individuals suspected to be infected with viral strains of that clade. For example, the IIIB (LAV) strain of HIV-1, first isolated in 1983 from an infected individual in the USA has been used extensively as a representative North American clade B strain virus to measure HIV-1-specific immunity among individuals infected with HIV-1 while in the USA.

Sequence identity of HIV-1 strains worldwide is between 85-97% (1), however, the greatest degree of diversity exists within the env gene of the virus. Sequence variation within the env gene can even be detected within viral variants sequenced from a single infected individual. These variants arise in an individual from continuous replication of the virus along the course of infection and have been detected using nucleotide sequence or heteroduplex mobility analyses (2). Diversification of the viral population during infection is a result of nucleotide substitutions, deletions, insertions or duplications within the viral genome which occur due to the low fidelity of reverse transcriptase and by viral recombination. The degree of nucleotide divergence over the entire genome among

isolates from one individual is approximately 6 to 10%. Within the env gene product itself, however, independent isolates have been demonstrated to differ in almost 10% of amino acid residues among North American patients and by as much as 26% among independent HIV-1 African isolates (3). The rate of evolution of the HIV-1 env gene in an infected individual has been estimated to be ten fold faster than that estimated for the gag gene (4). Furthermore, the nucleotide changes seen within the env gene of individual isolates involve insertions, deletions or duplications rather than the point mutations observed in the HIV-1 gag gene (1). The degree of heterogeneity within the env gene has been used to determine intrapatient and interpatient viral diversity.

The env gene product is a precursor polyprotein designated gp160 which becomes cleaved into gp120 and gp41, representing the surface and transmembrane subunits of the mature env glycoprotein complex, respectively. The precursor env protein is synthesized and glycosylated in the rough endoplasmic reticulum of the infected cell. Following synthesis, the glycoproteins oligomerize and are transported into the Golgi where they are cleaved into gp120 and gp41. It is also within the Golgi where many of the high mannose N-linked oligosaccharide side chains are modified. The resulting proteins are so heavily glycosylated that 50% of the molecular mass of gp120 is composed of oligosaccharides (1). A greater degree of variability is present within the gp120 encoding region of the gene in which five regions of relative conservation (C1-C5) are interrupted by five regions of hypervariability (V1-V5).

A high rate of HIV-1 replication throughout infection is needed to generate and maintain the complexity of the viral variant population observed in infected individuals. With the use of anti-retroviral drugs which strongly suppress viral replication, Wei et al (5) and Ho et al (6) demonstrated the rapid turnover of HIV-1 within the plasma of infected individuals. They demonstrated almost complete suppression of de novo infection by these anti-retroviral drugs which allowed them to calculate a 2 day half-life of the remaining

virion population within the plasma. Plasma virus was shown to derive primarily from de novo rounds of replication within a short-lived, unknown population of cells whose half-life could not exceed that of the virus. PBMC were therefore not considered to contribute significantly to this plasma pool of virus because they are longer lived. These studies showed that PBMC were turned over at a constant rate which suggested that progressive CD4 cell loss was due to continual de novo infection of PBMC. The maintenance of a viral load "steady state" in infected patients from a range of $10^2$ to $10^7$ virions per ml (7) must therefore require high levels of viral infection and production. It is this dynamic process of continual infection and replication which contributes to the generation of viral variants.

The evolution of viral variants also occurs from selective pressures within the host. A strong immune response against a particular region of a HIV-1 gene product might result in a selection of variants carrying within this region a mutation which can escape recognition. HIV-1 env, because it is a target of both cellular and humoral immunity as well as a determinant of viral tropism, is particularly affected by selective pressures. This is particularly applicable to the protein's V3 loop which contains at least three known CTL epitopes (8, 9) and is considered to be the principal neutralizing domain of the virus. Viral mutations have been reported within the V3 loop which weaken or abrogate CTL recognition (10-13) and recognition by neutralizing antibody (14). As an example of selective pressures applied by CTL on HIV-1 variation, nef CTL escape mutants were generated and disease progression was observed following the transfer of HIV-1 nef-specific autologous CTL to an AIDS patient (13). Following infusion of these CTL, nef variant sequences emerged with mutations in the CTL epitope which were accompanied by a rise in viral load.

The viral variants which evolve within an individual are not only genotypically distinct but also demonstrate phenotypic differences. These include replication rate, susceptibility to neutralizing and enhancing antibodies, tissue tropism and ability to induce

syncytia (15). In addition, studies in which viral variants have been analyzed at different points of infection suggest that the presence of particular phenotypes appears to relate to disease progression. For example, viral isolates taken from asymptomatic patients appear to replicate more slowly and to lower titers in tissue culture, whereas, viral isolates from AIDS patients appear to replicate more rapidly, to higher titers, to establish persistent infection in lymphoid and monocytoid cell lines and to induce syncytia formation.

In the majority of adults during primary infection and vertically infected infants within the first few months of life, the viral variant populations appear to be homogeneous in regard to both genotype and phenotype. Zhu et al (16) characterized the phenotypic and genotypic characteristics of HIV-1 variants within five seroconverters. The variants within each individual were phenotypically identical and were macrophage tropic and non-syncytia-inducing. In addition, env gene sequences from V3 to V5 of viral variants within each individual were greater than 99% similar compared with 90% to 94% similarity in the chronically infected transmitters. Delwart et al (17) using heteroduplex mobility and tracking analyses (2), also demonstrated a strong conservation of sequence within isolates present during primary infection. In addition, Wolinsky et al (18) sequenced the V3 and V4-V5 regions of several variants from vertically infants from two to four months of age. Similar to the findings in adults, these variants were relatively homogeneous.

## B. Clinical course of HIV-1

The clinical course of HIV-1 can be divided into two phases: a primary infection phase and a chronic phase. In primary HIV-1 infection, high levels of virus can be detected (7) in the plasma which reach a peak and then subside within weeks of infection and are maintained at a lower level. This subsequent lower level of virus is reported to range from $10^2$ to $10^7$ virions per ml of plasma (7) demonstrating that a high quantity of

virus is still maintained. The initial drop in plasma viremia, in adults, generally co-incides with the induction of humoral and cellular immunity. A clinically latent state ensues in which continual HIV-1 replication is accompanied by HIV-1 specific immune responses; particularly by CTL. This characterizes the chronic phase of HIV-1 infection. Eventually, in most patients, the CD4 pool of T lymphocytes becomes depleted with a concommitant severe immunodeficiency. The period of clinical latency is variable, but generally lasts for approximately 10-12 years. Recent reports have uncovered a population of individuals, however, in which CD4 depletion and disease progression have not occurred although they have been infected beyond the average amount of time within which one would expect to observe disease (19-22).

## C. HIV-1 specific immunity

Studies of cell-mediated HIV-specific immune responses of infected individuals reveal the presence of high levels of HIV-specific CTL activity within the peripheral blood of the majority of infected individuals (23-28). An unusual feature of HIV infection is the ease with which CTL activity against viral antigens can be measured during the asymptomatic period directly from freshly isolated PBLs in the absence of prior in vitro stimulation. High frequencies of CTL precursors have been quantitated from PBMC as well. Koup et al (29) quantitated HIV-1-specific CTL within the PBMC of patients during and three to six months following primary infection. The frequencies of HIV-1-specific CTL precursors were reported to be within a range previously reported in chronically-infected patients: from 30 to 3800 per $10^6$ PBMC (30-32). CTL activity has been demonstrated against both structural and nonstructural viral antigens (10, 33-35). The effector cell phenotype, characterized through antibody blocking experiments and depletion studies, is a CD8 and class I-restricted cytotoxic T cell. However, recognition of target

cells expressing the env gene appears to be mediated through both class I-restricted and non-HLA-restricted effector mechanisms, such as ADCC.

Two recent and separate studies provide compelling evidence for the role of CTL in primary infection. In these studies the development of HIV-specific CTL were examined during primary infection and correlated with viral load (29, 36). Both studies measured the viral loads and CTL precursors of HIV-1 infected patients from seroconversion to six months. Presence of HIV-1-specific CTL was associated with a decline in the intial viremia, whereas, lack of this CTL response appeared to correlate with higher viral loads. In addition to this recent data, prior studies have also demonstrated the ability of autologous CD8+ lymphocytes to inhibit HIV replication in PBL of infected individuals in vitro (37-39). Studies using an animal model also support the hypothesis that CTL may be protective in HIV infection. Multiple adoptive transfers of nef-specific CTL clones into PBL-reconstituted SCID mice (hu-PBL-SCID) just prior to and following HIV challenge was shown to protect against infection in a majority of the mice receiving these clones (40).

Humoral responses directed at particular regions of the env gene can direct virus neutralization. The primary neutralizing domain of env is considered to be the V3 loop. Antibodies directed to this region in infected individuals have been able to neutralize heterologous strains of HIV-1 in vitro (41-43). However, monoclonal antibodies against the CD4 binding domain within C4 (44, 45), regions within V2 (46)and discontinuous epitopes within env which can(47, 48) and cannot inhibit CD4 binding (49) have also been shown to neutralize the virus in vitro.

Animal models of HIV-1 infection provide evidence for a possible role of neutralizing antibody for protection when passively transferred prior to infection (50). However, there does not appear to be a strong correlation between the presence or absence of these antibodies and protection in natural infection. Neutralizing antibody responses have been reported to occur shortly after seroconversion and appear to be isolate-specific

(51). Neutralizing antibody may therefore contribute to the clearing of early viremia. The specificity of this response apparently broadens over time as antibody which can neutralize heterologous laboratory strains can be detected although these antibodies are present at low titers. It has been demonstrated that with the progression of infection within an individual, antibodies may be capable of neutralizing laboratory strains of HIV-1, while they may not be able to neutralize autologous virus (14, 51). The presence of neutralization escape mutants implies that neutralizing antibodies are exerting selective immune pressure in vivo. It therefore seems likely that this response serves a protective role although no clear correlation has been made between its presence and viral load.

The env gene of HIV-1 can also serve as a target for ADCC. This is an immune mechanism whereby lysis of infected cells is effected by the binding of antibody attached to NK cells via the Fc receptor to the env glycoprotein on the surface of the cell. This is, therefore, a humoral response directed solely at cell-associated virus. ADCC-mediating antibodies have been demonstrated in HIV-1 infected individuals (52-54). ADCC antibody epitopes have been mapped to both gp120 and gp41(55, 56).

The role of ADCC antibody in control of HIV-1 replication is perhaps the least well-defined. The most notable example of a protective role for ADCC in viral infection is a murine model of vertical transmission of Herpes Simplex Virus. Transfer of ADCC mediating antibody into neonatal mice in the presence or absence of murine or human effector cells resulted in protection of HSV-susceptible mice (57, 58). ADCC antibodies in adult humans may also play a role in controlling early viremia in HIV-1, as they are of the first humoral responses to be detected (59) and demonstrate broader reactivity prior to that of neutralizing antibody. However, ADCC antibody effectiveness has not been extensively studied in HIV-1 infection. ADCC could be important in the elimination of cell-associated virus and could work in concert with CTL in this endeavor.

## D. Vertical transmission of HIV-1

The majority of HIV-1-infected children have become so through vertical (maternal) transmission of the virus. Within the United States alone, HIV-1 infection is the seventh leading cause of death among children 1 - 4 years old and ranks much higher in developing countries (60).

Transmission of HIV-1 to the fetus can occur during gestation. The mechanism of in utero transmission is not well understood. One hypothesis is that a placental disruption, such as infection, can allow for the contact of maternal and fetal lymphocytes (61, 62). Hofbauer cells, which are monocytes of fetal origin located on the fetal side of the placenta, have been theorized to be a target for infection. Another possibility is that IgG complexed with virus is allowed entry into the fetal circulation through transplacental transfer of maternal immunoglobulin. Transmission can also occur during birth. During labor and delivery, mucosal surfaces of the neonate are exposed to maternal blood and cervico-vaginal secretions. Postpartum transmission of HIV-1 can also occur through breast milk. Breast milk transmission is less likely to occur in the United States where, if HIV-1 positivity is known, mothers are counselled to avoid breast feeding.

Diagnostic standards have been agreed upon to determine the timing of HIV-1 infection in infants (63). Detection of virus by culture or DNA PCR within 48 hours of birth with additional positive cultures and DNA PCR subsequent to this time defines the infection as having occurred in utero. PBMC from infants which are negative for viral culture and PCR within the first week of life but subsequently are repeatedly positive are considered to have been infected in the intrapartum period.

Similar to the adult pattern of acute infection, infants experience a burst of viremia which occurs between the third and sixteenth week of life, regardless of the timing of

infection. However, unlike adult infection, the majority of infants undergo a more rapid course of disease progression than adults. In a period between 1981- 1992, greater than 44% of perinatally infected infants had AIDS diagnosed within their first year of life as reported to the Centers for Disease Control (63)

## E. Infant HIV-1 specific immunity

1. CTL. Detection of HIV-1 specific CTL in early infancy is rare and usually requires in vitro stimulation of PBMC. After 12 months of age, CTL responses become more prevalent, but, the detection still requires in vitro stimulation in a majority of children. This is in direct contrast to CTL detection in adults. Luzuriaga et al (64), measuring HIV-1 gag-specific CTL directly from peripheral blood taken from 12 vertically infected infants 6 to 30 months of age, detected responses in only 3 of these individuals. Gag-specific CTL responses were not detected in these individuals before 12 months of age. Buseyne et al (65) measured cytolytic responses in HIV-1 infected children of whom the majority were greater than 12 months of age. Low level HIV-1 specific cytolysis was detected directly in freshly isolated PBMC of 70% of asymptomatic children. Only 20% of clinically progressed children demonstrated CTL responses in these primary assays. In vitro stimulation of these same PBMC using PHA and IL-2 for a period of 15-30 days resulted in cytolytic activity against HIV-1 within all of the patients of both populations. Froebel et al (66) studied a cohort of children of similar age and clinical status in which they detected HIV-1 specific CTL following 9-23 days of in vitro stimulation using PHA and IL-2. McFarland et al (67) also detected CTL activity directly from PBMC in only a small percentage of children over 12 months of age, but following nonantigen-specific in vitro stimulation, were able to quantitate the number of CTL precursors within this group. These children demonstrated gag- and env-specific pCTL frequencies from 50-630/$10^6$

PBMC and from 66-3300/$10^6$ PBMC, respectively. Studies within our laboratory, have focused upon the CTL response within the first year of life. The majority of the infants who were below 12 months of life lacked detectable HIV-1 specific CTL responses within unstimulated PBMC (68). Although nonviral-specific stimulation allowed for detection of CTL in some of these patients, others still lacked measurable CTL responses even after in vitro stimulation. A notable finding within this study was the detection of gag-specific CTL precursors in the cord blood of one infant on the order of 333 pCTL/$10^6$ PBMC. This finding demonstrates the possibility of fetal immune responses.

It is notable that all of the studies described above employed targets expressing heterologous HIV-1 gene products derived from laboratory strains. The CTL recognition and cytolysis of targets expressing HIV-1 protein derived from autologous virus has not been addressed.

2. Neutralizing antibody. One cannot distinguish between IgG antibody of maternal or infant origin within the first six months to a year of life. Therefore, neutralizing antibody titers within the mother during pregnancy have been examined extensively in an attempt to find a correlation between transmission and neutralizing antibody titers. Husson et al (69) examined the transmission rate and maternal neutralizing antibody titers against autologous maternal and infant viral isolates in a population of women matched by CD4 percentage and use of zidovudine during pregnancy. Results of this study indicated a positive correlation between low titers of autolgous neutralizing antibody and an increased risk of transmission. Scarlatti et al (70) demonstrated similar results. Neutralizing antibody titers from sera from HIV-1 infected mothers were quantitated against autologous and clinically-derived heterologous virus. Neutralizing antibodies against autologous virus were more frequently detected in nontransmitting women in this study. In addition, women who had measurable titers of neutralizing antibody to autologous virus also were able to neutralize at least two heterologous viruses.

Broliden et al (54) studied neutralizing antibody titers in 0 - 2 year old children in an attempt to correlate these titers with clinical outcome. Neutralizing titers against heterologous viral strains (IIIB and RF) were highest in patients who lacked any AIDS defining condition. Results of this study also demonstrate a lack of detectable neutralizing antibody in these infants following loss of maternal antibody. However, neutralization was not measured against autologous virus.

3. ADCC antibody In an attempt to correlate additional HIV-1-specific humoral responses to the risk of vertical transmission, ADCC antibody titers have been quantitated within infected transmitting mothers and compared with nontransmitters. Jenkins, et al (71), measured ADCC antibody titers against HIV-$1_{IIIB}$ and -$_{RF}$ strains in mother-infant pairs. Results of this study indicated that high levels of ADCC antibody did not correlate with a decreased risk of transmission. Broliden, et al (54), also examined ADCC antibody titers to HIV-$1_{IIIB}$ and -$_{RF}$ in an attempt to correlate clinical outcome of HIV-1 infection with these titers. ADCC antibody was observed more frequently in non-AIDS infants than in those with AIDS. Results of their study also demonstrated a lack of ADCC antibody against heterologous strains of HIV-1 following clearance of maternal antibody. Using IIIB env expressing targets, we have also observed a lag in ADCC antibody lysis until at least two years of age in HIV-1 vertically infected infants following clearance of maternal antibody. ADCC titers have not, however, been quantitated against autologous virus where stronger correlations between both transmission and progression may be evident.

4. Summary of HIV-1-specific immunity in vertically infected infants On the basis of HIV-1-specific immune responses against laboratory strains of HIV-1, vertically infected infants appear to be deficient in CTL responses within the first year of life and ADCC antibody and neutralizing antibody responses within at least the first two years of life. Table 1 is a comparison of HIV-1-specific immunity in adults and vertically infected infants.

F. Hypotheses to explain lack of HIV-1 specific immunity in early infancy

Induction of tolerance Tolerance induction as a result of in utero or intrapartum aquisition of HIV-1 infection is a possible explanation for the lack of detectable HIV-specific CTL during early infancy. A murine model of tolerance induction to viral antigen is provided by that of mice exposed to lymphocytic choriomeningitis virus (LCMV) either in utero or as neonates. LCMV-specific humoral immunity occurs during infection, but LCMV-specific CTL cannot be detected following antigenic stimulation of spleen cells in vitro, and a LCMV carrier state is maintained (72). Tolerance to LCMV can be reversed in these animals if they are cleared of virus through adoptive transfer of LCMV-specific CTL from immune animals. Upon subsequent challenge with LCMV, these animals mount a strong LCMV-specific cell mediated response. Tolerance induction may rely on factors other than timing of transmission, such as the differing affinities possessed by different HLA class I alleles for HIV-1 antigen. Von Herrath et al (73) has demonstrated an HLA class I allele-specific influence on thymic selection of LCMV-specific CTL. In this study, H-$2^d$ and H-$2^b$ mice were made transgenic for the NP gene of LCMV. This gene, under expression of the Thy1.2 promoter, was expressed in the thymus and peripheral T lymphocytes of these mice. H-$2^d$ mice deleted their high affinity anti-LCMV-NP CTL but generated equal number of lower-affinity CTL of NP specificity. In contrast, H-$2^b$ mice deleted all LCMV-NP-specific CTL, completely abrogating detection of these CTL.

Inability to detect type-specific immunity with heterologous virus HIV-1 specific CTL and infant-derived ADCC and neutralizing antibody may be present within the first year of life in vertically infected infants which have gone undetected due to assay limitations. Most detection systems used to measure HIV-1 specific immune responses utilize genes from laboratory virus strains. Immune responses may be directed only to the

fairly homogeneous variant population observed in early infection. Lack of group-specificity of cellular and humoral immune responses may account for their observed absence in early infancy. Env responses would be expected to be the most type-specific of all due to its variability between HIV-1 strains.

Immaturity or dysfunction of immune response The infant's immune system may not be sufficiently developed at the time of infection to mount a virus-specific immune response. The presence of high levels of CTL precursors in cord blood which we have previously reported (68) presents a strong argument against this hypothesis unless immune maturation was somehow linked to a differential effect of HIV-1 viral replication in individual patients. Valentin, et al (74) have proposed a mechanism whereby both CD4 and CD8 cells can be depleted in infants infected in utero. They demonstrate that triple-negative (CD3- CD4- CD8-) thymocytes are susceptible to infection. Infection of these cells as well as double positive cells (CD4+ CD8+) could lead to an overall defect in T cell function in these patients. A deficit in the ability of neonatal lymphocytes to proliferate and produce interferon-gamma in response to specific antigenic stimulation has been observed in primary herpes simplex virus infection (75). Furthermore, neonatal lymphocytes produce reduced levels of IFN-γ to a number of different stimuli (76, 77) due to a decrease in transcription of the IFN-γ gene (78). Memory T cells, a subset of responding lymphocytes, are responsible for high levels of IFN-γ production upon antigenic stimulation (79). The low levels of IFN-γ produced by neonates may be due to the absence of this subset of high T cell responders. Low CD40 ligand expression on neonatal T cells has also been reported (80). The CD40 ligand is a molecule responsible for ligating CD40 on the B cell to provide T cell help in immunoglobulin isotype switching. However, with antigen stimulation in vitro, expression of this ligand increases, suggesting that the lower expression in vivo is due to a antigen-naive population of T cells. A diminished ability of infant NK cells, in comparison to adults, to mediate ADCC against HIV-infected target

cells has also been reported (71). There are conflicting data regarding the functional maturity of cord blood lymphocytes. Harris et al (81) report that cord blood lymphocytes do not repond well to in vitro stimulation using IL-2, PHA or alloantigens. On the other hand, Stern et al (82) demonstrate PHA responsiveness within the lymphocytes of cord blood which is equivalent to that seen in older infants and adults. For that matter, PWM and ConA responsiveness in cord blood appeared to be more vigorous than that seen at 10 months of age. The responses at 10 months of age to PWM and ConA were equivalent to those of adults. The percentages of total T cells, T-cell subsets and B cells increased from birth to later infancy and the CD4/CD8 ratio decreased. The percentage of CD8 cells was lower in 10 month old infants in comparison with that of adults, therefore the CD4/CD8 ratio was still higher at this age than that of adults.

## G. Objectives of this study

The aim of this study was to determine whether infants vertically infected with HIV-1 are capable of mounting an HIV-1-specific immune response against autologous virus. The experimental design employed to achieve this objective involved the measurement of both cellular and humoral immunity through quantitation of CTL and ADCC responses. These experiments were undertaken to determine whether the previously observed inability to detect HIV-1 specific CTL and ADCC responses within the first year of life is due to type-specific immune responses or a complete lack of HIV-1 specific cellular and humoral immunity.

Secondly, if autologous virus-specific responses were present, a second objective was to examine the patterns of responsiveness over time to two sequential isolates and to examine the T cell responsiveness on a clonal level. Evaluation of patterns of

responsiveness could possibly provide clues as to how the immune response differs in vertically infected infants in comparison to infected adults.

HIV-1-specific immunity may differ in infants infected in utero vs those infected during the intrapartum period due to a less mature immune system at the time of infection and the possibility of neonatal tolerance. Therefore, a final objective was to determine whether a difference existed in the capabilities of in utero versus intrapartum infected infants to mount HIV-1 specific immune responses.

Table 1. HIV-1 specific immune responses against laboratory strains of HIV-1 in adults and vertically-infected infants and children

| | *ADULT* | | *INFANT (< 12 MO)* | | *CHILD (> 12 MO)* | |
|---|---|---|---|---|---|---|
| CTLp | +++++ | (29-32) | + | (68) | ++++ | (67, 68) |
| Bulk CTL | +++++ | (23-27) | +/- | (68) | + | (65, 68) |
| ADCC antibody | +++++ | (52, 53) | +++ | (54, 71) | + | (54) |
| Neutralizing antibody | ++ | (83, 84) | ++ | (54, 69, 70) | + | (54) |

# CHAPTER II. MATERIALS AND METHODS

## A. Patients:

HIV-1-specific CTL responses were studied retrospectively in 4 HIV-1 infected infants whose clinical status was followed at the University of Massachusetts Medical Center. The timing of HIV-1 infection was determined by previously established standards (85). Patients VI-05 and VI-06 whose cord or neonatal lymphocyte samples were positive for HIV-1 by viral culture and/or DNA PCR were considered to have been infected in utero. Patients VI-08 and VI-11 were considered to have been infected during the intrapartum period. Lymphocyte samples were negative for HIV-1 by viral culture and DNA PCR prior to a month of age in these two patients. All four patients demonstrated HIV-1 positivity upon subsequent repeated testing. Analyses of CTL responses against the env and gag gene products of the IIIB strain of HIV-1 have previously been presented on these four infants (68). Human Studies Committee approval and individual informed consent from each infant's guardian were obtained before we conducted these studies. Table 2 describes the ages at which env genes were amplified from viral isolates and the virological and clinical status of each patient.

## B Reagents:

1. Plasmids: table 3

2. Primers: table 4 and 5

Table 2. Time points at which env genes were derived from viral isolates and clinical and virological status of patients VI-05, VI-06, VI-08 and VI-11.

| Patient, Age at each time point | CD4% | Absolute CD4 | RNA* | PBMC culture‡ | Drug therapy |
|---|---|---|---|---|---|
| VI-06, 1 d | 15 | 1404 | 49,000 | 25 | no |
| VI-06, 6 mo§ | 37 | ND | 70,000 | ND | yes |
| VI-05, 2 mo | 20 | 3015 | ND | 625 | no |
| VI-05, 6 mo | 24 | 1895 | ND | 25 | no |
| VI-08, 20 d | 50 | 2091 | ND | 125 | no |
| VI-08, 6 mo | 36‖ | 2547‖ | ND | ND | no |
| VI-11, 1 mo | 29 | 1754 | ND | ND | no |
| VI-11, 3 mo | 34 | 2218 | ND | 0 | no |

* RNA copies/ml plasma

‡ $TCID_{50}/10^6$ PBMC

§ env clones were derived from this time point but env recombinant vv were not constructed

‖ data available only at 5 months of age

ND not done

Table 2. Time points at which env genes were derived from viral isolates and clinical and virological status of patients VI-05, VI-06, VI-08 and VI-11.

HIV-1 was cultured from infected PBMC from patients VI-05, VI-06, VI-08 and VI-11 at the time points described. Supernatant from these cultures was used to establish infection in donor PBMC and genomic DNA was extracted and used as a template for PCR amplification of the HIV-1 env gene as described in the Materials and Methods section. The clinical status of the patient at these time points is represented by the CD4 percentages and absolute CD4 values. Virological status is represented by the viral RNA copy number.

Table 3. Plasmids

| *Plasmid* | *Description* |
|---|---|
| pNL4-3 | full-length molecular clone of HIV-1; chimeric: 5' half is NY5 and 3' half is LAV |
| pCR3 | InVitrogen; eukaryotic expression vector; TA cloning strategy; CMV promoter |
| pT2020 | Therion Biologics; pAbT4587 backbone with HIV-$1_{MN}$ env gene |
| pAbT4587A | Modified version of pAbT4587 (XhoI site in place of EcoRI) |
| pAbT4587 | Therion Biologics; contains flanking nonessential vac sequences on either side of cloning site; used to make recombinant vaccinia virus |
| pAbT4603 | Therion Biologics; pAbT4587 backbone with HIV-1 BH10 env gene |

Table 4. Primers for PCR:

| *Primer Designation* | *Sequence* | *Annealing Position** |
|---|---|---|
| MNA | GCGAAAGAGCAGAAGACAGTGGC | 6197-6220 |
| MN13 | CAGCTCGTCTCATTCTTTCCC | 8836-8857 |
| 209 | ACCCCAACCCACAAGAAG | 6453-6470 |
| 218 | CTGTTTAATAGTACTTGG | 7382-7399 |

* Oligonucleotide sequences described anneal within the HIV-1 genome at positions based upon published pNL4-3 nucleotide positions (86).

Table 5. Primers for Sequencing:

| *Primer Designation* | *Sequence* | *Annealing Position** |
|---|---|---|
| SP6 | CATACGATTTAGGTGACACTATAG | SP6 promoter |
| T7 | TAATACGACTCACTATAGGGAGA | T7 promoter |
| MNA | GCGAAAGAGCAGAAGACAGTGGC | 6197-6220 |
| 210 | GGTTGGGGTCTGTGGGTA | 6444-6461 |
| 209 | ACCCCAACCCACAAGAAG | 6453-6470 |
| 212 | TTATCTCTTATGCTTGTG | 6706-6723 |
| 214 | GGCAGTCTAGCAGAAGAA | 7001-7018 |
| 215 | TCTGGGTCCCCTCCTGAG | 7306-7323 |

* Oligonucleotide sequences described anneal within the HIV-1 genome at positions based upon published pNL4-3 nucleotide positions (86).

C. Amplification of HIV-1 proviral DNA from infant isolates:

1. Viral Culture: Viral co-culture was set up from PBMC samples taken from patient VI-06 at one day and 6 months of age, patient VI-05 at 2 months and 6 months of age, patient VI-08 at 20 days and 6 months of age and patient VI-11 at one month and three months of age. Supernatants from these cultures were used to establish first passage viral cultures in the following manner: 10 x $10^6$ donor PBMC grown in 10% FCS and RPMI and stimulated for 5 days with 5 U/ml IL-2 were pelleted and resuspended in 1 ml culture supernatant and incubated at 37°C, 5% $CO_2$ for 1 hour. The cells were washed to remove input virus and resuspended in 10 ml RPMI, 10% FCS, 5 U/ml IL-2. Culture supernatant was sampled on day 3 and 5 and analyzed for p24 content. Second passage viral cultures were also established in the same manner.

2. Genomic DNA extraction: Approximately 10 x $10^6$ cells from the viral cultures were pelleted from low passage viral cultures on all patients. Cells were then washed twice with PBS and resuspended in 5 ml lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, pH 8.2) to which was added 10% SDS to a 0.7% final concentration and 1.7 mg proteinase K. The lysed cells were incubated at 37°C overnight. After overnight incubation, DNA was obtained following this incubation by adding 1.6 ml NaCl, centrifuging for 15 min at 2500 rpm and adding 7 ml iso-propanol to the supernatant. The DNA was pelleted by centrifugation at 3500 rpm for 20 min, washed with 70% ethanol and stored at -20°C until PCR was performed.

3. PCR: Approximately 200 ng of genomic DNA from each of the eight cultures described above were used to amplify a 2.6 kB product of HIV-1 proviral sequence containing the entire coding sequence for gp160. Reaction conditions were 2.5mM $MgCl_2$, 200 uM of each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3 (GeneAmp PCR Buffer II -

Perkin Elmer), 10pM of primers MNA and MN13 and 2.5U AmpliTaq (Perkin Elmer). PCR reactions were initially melted for 3 min at 94°C, followed by addition of AmpliTaq during an 87°C hold. The cycling program consisted of 30 cycles of the following: 1 min denaturation at 94°C, 1.5 min annealing at 60°C and 3 min extension at 72°C, followed by a final 10 min extension at 72°C. In a nested amplification of a smaller fragment of the env gene, the first PCR reaction was spun through CL-4B sepharose. Ten percent of the first reaction was used as template in a second amplification to yield a 964 bp fragment of env containing V1-V3. This was amplified using primers designated 209 and 218. Env clones were used as templates to generate $^{32}P$ internally labeled PCR products using $^{32}P$-dCTP in the dNTP mix according to the methods of Delwart, et al (2). PCR conditions using primers 209 and 218 were the same as those for MNA and MN13 described below except for a $MgCl_2$ concentration of 4mM instead of 2.5mM. Cycling conditions were the same except for the absence of a hot start and an annealing temperature of 55°C instead of 60°C.

D. Divergence analysis of patient provirus from HIV-1IIIB using heteroduplex formation:

1. Heteroduplex formation: Heteroduplexes were formed between the labeled and unlabeled PCR products as described by Delwart, et al (2). Briefly, PCR products were combined in a ratio of 1:100 labeled:unlabeled in 100mM NaCl, 10mM Tris-HCl (ph 7.8) and 2 mM EDTA in a total volume of 10ul. This mixture was heated at 94°C for 2 min. and then rapidly cooled on ice.

2. Gel electrophoresis: DNA fragments were separated in a 5% polyacrylamide gel (30:0.8 acrylamide: Bis) in TBE buffer (0.088M tris-borate, 0.089M boric acid, 0.002 M EDTA, pH 7.0) at 30mA for 12 hr. The gel was then dried for 1 hr at 80°C and exposed to radiographic film.

E. Vaccinia Vector Construction:

1. Ligating env gene into pCR3 (InVitrogen) Approximately 30% of the PCR products were electrophoresed on a 0.8% agarose gel and the 2.6kB fragment from each was excised and cleaned with the gel purification kit Eluquick (Schleicher & Schuell). The addition of dATP to the 3' ends of PCR products carried out by Taq polymerase was utilized to ligate these products into a eukaryotic expression vector, pCR3 (InVitrogen) which possesses overhanging dTTPs on its 5' ends. Fifty ng of pCR3 was ligated with approximately 120 ng of the gel purified PCR product to achieve an insert:vector ratio of approximately 4:1 using 1ul T4 DNA ligase (InVitrogen) in a ligation reaction of 10ul total volume. The ligation reaction was incubated at 16°C for 3 hrs. Two ul of the ligation reaction was used to transform 50ul competent TOP10F' cells (InVitrogen) in the following manner: 50ul of TOP10F' cells previously stored at -80°C were thawed on ice and mixed with 2 ul of the ligation reaction. This was incubated for 20 min on ice and then heat shocked at 42°C for exactly 45 sec. Three hundred and fifty ul of LB media were added and this was incubated on ice for 2 min. One hundred ul of this was plated onto LB agar plates containing 100ug/ml carbenicillin (SIGMA) and incubated overnight at 37°C.

2. Preparation of plasmid DNA for screening Ten colonies were selected for screening from ligations involving the following env's: Patient VI-06 at 6 months of age; patient VI-08 at 20 days and 6 months of age; Patient VI-05 at 2 months and 6 months of age. Twenty colonies were selected and screened from ligations involving env's from patient VI-11 at 1 month and 3 months of age and patient VI-6 at 1 day of age. These colonies were inoculated into 3 ml of LB containing 100 ug/ml carbenicillin and grown at 37°C over night. Plasmid DNA was isolated using an acid-phenol miniprep to render the

DNA clean enough for sequencing. Acid phenol is used in place of phenol chloroform in an alkaline lysis procedure after precipitation of cell debris and SDS with potassium acetate. The aqueous layer is further extracted with chloroform/isoamyl alcohol (24:1, vol:vol) followed by conventional ethanol precipitation.

3. Screening of plasmids: a. Restriction Digests: approximately 1 ug of DNA from these preps was used to determine whether the plasmid contained an env insert in 20 ul restriction digests using 1 ul EcoRI which cleaves on both sides of the cloning site of pCR3. b. Sequencing: 1. To determine orientation: all plasmids containing the 2.6kB insert were sequenced to determine their orientation within pCR3 in the following manner: Approximately 1 ug of DNA was denatured using 200 mM NaOH and .2 mM EDTA in the presence of 70ng of either T7 or SP6 primer (InVitrogen). The denatured DNA was precipitated with 200 mM sodium acetate pH 4.5 and 3 vol ethanol, washed with 70% ethanol and dried for 2 min. in a speedvac. The DNA pellet was resuspended and sequenced according to the Sequenase 2.0 protocol (USB) using $^{35}$S-dATP (NEN). 2.5 ul of these reactions were analyzed on a vertical polyacrylamide gel containing 6% acrylamide and urea in TBE buffer using the Model S2 Sequencing Gel Electrophoresis Apparatus (Gibco BRL). Briefly, the sequencing reactions were heated for 2 min at 85°C and then electrophoresed at 40mA for 1.5 hr. The gel was then dried for 1 hr at 80°C, exposed to radiographic film for approximately 12 hr., developed and read. 2. CTL epitope analysis: All of the clones obtained from patients VI-06 and VI-08 were sequenced with primers MNA, 210, 209, 212, 214 and 215. These are regions within env that contain sequences published as CTL epitopes restricted by HLA-A2 (8, 87). Translation of these nucleotide sequences was performed using MacVector (Eastman Kodak).

4. Grouping of env clones: Plasmids containing the env insert from each patient at each time point were digested with StuI (NEB), AvaI (NEB), BamHI (Promega), KpnI (Promega), BglI (Promega) and HindIII (Promega). The presence of distinct groups of

clones could be observed within env clones derived from each patient at each time point by the appearance of distinct restriction digest patterns. Each clone was categorized into one of the groups represented by a particular restriction digest pattern. Based upon these restriction patterns, the most commonly represented env clone was chosen to be subcloned.

5. Subcloning of env genes into pAbT4587A: a. Competent E. coli DH5α: E. coli strain DH5α were made competent using a conventional calcium chloride protocol. b. Creating XhoI site in pAbT4587 (Therion): The pAbT4587 plasmid from Therion was modified in order to create appropriate restriction sites for directional subcloning of the env genes ligated into pCR3. The previously existing EcoRI site was destroyed by EcoRI digestion of 1 ug of pAbT4587 followed by incubation with 1 ul Klenow fragment and 125 uM each dNTP at room temperature for 30 min. The plasmid DNA was then extracted once with phenol chloroform and ethanol precipitated and used in a ligation reaction with 1 ug of non-phosphorylated XhoI linker (New England Biolabs) and 1ul of T4 DNA ligase (Promega). The ligation reaction was allowed to proceed at room temperature for 1 hr. Half the ligation reaction was used to transform 100 ul competent E. coli strain DH5α and colonies were grown overnight at 37°C on LB plates containing 100ug/ml carbenicillin. Ten colonies were selected and minicultures were set up as previously described. Plasmid DNA was isolated from these cultures using a conventional alkaline lysis miniprep protocol. Approximately 1 ug of DNA was used to screen for the presence of the XhoI site by a double restriction digest using 1ul XhoI and 1 ul HindIII. The modified plasmid, designated pAbT4587A, was amplified in a 250 ml culture of LB/carbenicillin and maxiprepped and purified using the Qiagen maxiprep kit. c. Ligation of selected env genes into pAbT4587A: One env gene was selected from each set of clones from each patient at each time point as previously described for a total of 8 env clones. In each case the env gene was in the correct orientation for expression by the CMV promoter within pCR3. Each env gene was excised from the pCR3 backbone using BamHI and XhoI which are

located within the pCR3 cloning site, 5' and 3' of the env gene, respectively. The 2.6kB BamHI-XhoI fragment was gel purified using Eluquick (Schleicher & Schuell) and ligated into the BamHI-XhoI fragment of pAbT4587A at an insert:vector ratio of 4:1 as previously described. Competent E. coli strain DH5a were transformed and colonies selected and minicultured as previously described. Plasmid DNA was prepared from 6 separate colonies derived from each ligation using a conventional alkaline lysis protocol and screened for the presence of the relevent insert by a double restriction digest using BamHI and XhoI. One plasmid from each ligation positive for the 2.6kB insert was amplified in 250ml LB/carbenicillin and maxiprepped with the Qiagen maxiprep kit. d. Analysis of subcloned env's using HTA: $^{32}P$ labeled-PCR products of 964 bp spanning V1-V3 as described above were generated from the env genes ligated in the pCR3 backbone selected for subcloning. These were then used as probe against PCR products derived from their subclones (ligated into pAbT4587A) as previously described. A second probe, the V1-V3 PCR product of pNL4-3, was also used to probe the subcloned envelope genes.

Table 6. Patient-derived env clone designations.

| *Patient* | *clone name at 1st time point in pCR3* | *clone name at 1st time point in pAbT4587A* | *clone name at 2nd time point in pCR3* | *clone name at 2nd time point in pAbT4587A* |
|---|---|---|---|---|
| VI-06 | pCAR-1D | pTAR-1D | pCAR-6M | ND |
| VI-05 | pCHE-2M | pTHE-2M | pCHE-6M | pTHE-6M |
| VI-08 | pCJA-20D | pTJA-20D | pCJA-6M | pTJA-6M |
| VI-11 | pCRO-1M | pTRO-1M | pCRO-3M | pTRO-3M |

ND: not done due to lack of stablity of env gene from this time point in this plasmid

Env genes were amplified by PCR and cloned into pCR3 (InVitrogen) and have the designation "pC" preceding the clone name. One representative env clone from each set of clones derived from each time point from each patient was chosen due to its predominance (determined by restriction digest). The env gene from this clone was subcloned into the pAbT4587A plasmid and the resulting subclone has the designation of "pT" preceding the clone name.

6. Transient Expression Analysis a. Infection of RK-13 cells with vaccinia: Monolayers of RK-13 cells (ATCC # CCL37) grown in DMEM, 10% FCS in 6-cm dishes were infected with wild type vaccinia virus vAbT33 (Therion Biologics) at an MOI of 1 in DMEM, 2% FCS. These were incubated at 37°C, 5% $CO_2$ for 45 min. b. Transfection of vaccinia infected RK-13 cells: At the end of this incubation, the vaccinia-infected cells were transfected with 20 ug of each env clone described above using a conventional calcium phosphate transfection method (88) and incubated at 37°C, 5% $CO_2$ for 16 hrs. c. Cell staining: Following the 16 hr incubation, cells were fixed in formaldehyde and incubated for 1 hr at 37°C with a serum pool from rabbits immunized with env protein from HIV-1 $_{IIIB}$ and HIV-1 $_{SF-2}$,which was diluted 1:500 in phosphate buffered saline (PBS) and 50% normal goat serum. Cells were then washed and incubated with 1:200 goat anti-rabbit IgG (H&L) (Perry & Kirkegaard Laboratories) conjugated to alkaline phosphatase for the same amount of time. Cells were then washed and 1 ml of BCIP/NBT substrate/developer (P & L Laboratories) was added. The reaction was allowed to proceed for approximately 5 minutes and was stopped by the addition of 2 ml PBS. Expression of env was determined by the presence of blue-stained cells.

7. Recombinant vaccinia generation: a. *In Vitro* recombination: *In vitro* recombination and recombinant vaccinia generation were performed according to the methods detailed by Mazzara et. al. (88). The infection and transfection of RK-13 cells used to achieve recombination between wild type vaccinia and the env clones was as described above with the exception that the cells are incubated for 48hr instead of 16hr. b. Selection of recombinants: When the cells demonstrated 100% CPE at 48 hr, the medium was removed and the cells were scraped into 1mM Tris-HCl, pH 9.0 and freeze/thawed 3 times to release virus. Ten-fold serial dilutions were prepared from the IVR-generated virus. $10^{-1}$ to $10^{-4}$ dilutions were plated on confluent monolayers of RK-13 cells in 6-cm

dishes in media conditions described above and allowed to adsorb for 30 min. Following adsorption a 0.6% agarose-DMEM-5%FCS overlay was added. After a 48 hr incubaton at 37°C, 5% $CO_2$, another overlay was added containing 0.4 mg/ml Bluogal (SIGMA). This was incubated for another 48 hr. at which time 8 white plaques were selected from each IVR and stored at -80°C. Confluent monolayers of RK-13 cells in 6-cm dishes were infected with 5ul and 10ul of one of each set of plaque isolates for further purification. Agarose overlays were added as previously described. If no blue plaques were present at this second step, vaccinia recombinants were considered to be pure from the first plaque isolate. If blue plaques appeared, white plaques were again selected from the second round isolates and underwent a third round of purification to ensure purity. Pure plaques were then amplified on confluent RK-13 cell monolayers in 6-cm dishes and virus was obtained 48 hr after infection by the freeze-thaw method described above.

8. Analysis of expression using a radioimmunoprecipitation assay: Confluent monolayers of CV-1 (ATCC# CCL70) cells in 6-cm dishes were infected with 400 ul of crude virus prep from each IVR as described above and simultaneously labeled with 100 uCi $^{35}$S-met;$^{35}$S-cys (NEN) in methionine and cysteine free DMEM, 4% FCS for 16 hr at 37°C, 5% $CO_2$. Cells were then scraped into RIPA buffer containing 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM Tris-HCl (ph 7.5), 0.25M NaCl and 5mM EDTA (88). Cell lysates were split and incubated for 2 hr at room temperature with pooled sera diluted 1:200 from HIV positive or HIV negative individuals. Immune complexes were then precipitated with protein A-sepharose for 90min at room temperature. The beads were washed several times in RIPA buffer and the immune complexes were removed from the beads through a 10 min incubation at 100°C. An equal volume of 2X Laemmli sample buffer was added to each immunoprecipitate and loaded into the wells of a denaturing 6% Laemmli polyacrylamide gel. The gel was run at 150 mA for

4 hr, fixed, enhanced with Amplify (Amersham), dried at 80°C for 2hr and exposed to radiographic film.

9.Amplification, purification and titration of recombinant vaccinia virus: a. amplication: confluent monolayers of RK-13 cells in 6-cm dishes were infected with 400ul of crude virus preparation and incubated until 100% CPE were obtained (approximately 48 hr). These cells were scraped into 1mM Tris-HCl, pH 9.0 and freeze-thawed three times. All of this crude virus preparation was used to infect a confluent monolayer of RK-13 cells grown in a T-175 tissue culture flask (approximately 15 x $10^6$ cells) and incubated at 37°C, 5% $CO_2$ until 100% CPE was demonstrated. Cells were scraped into 5ml Tris-HCl and virus released as described above. b. Purification on sucrose cushion: virus was purified according to the method of Mazzara et al (88) . Briefly, the 5 ml of amplified virus was layered on 15 ml of 36% (w/v) sucrose in 1 mM Tris-HCl, pH 9.0, in a 30-ml Beckman ultracentrifuge tube for the SW28 rotor. These were then centrifuged for 1 hr at 20,000 rpm at 4°C to pellet the virus. The viral pellet was resuspended in 1 ml of 1 mM Tris-HCl, pH 9. c.Titering of purified virus: virus was titered according to the method of Mazzara et al. (88) with slight modification. Briefly, confluent monolayers of CV-1 cells in 6-well plates were infected in duplicate with 0.4 ml of 10-fold serial dilutions of purified virus over a range of $10^{-6}$ through $10^{-11}$ dilutions. The cells were incubated for 48 hr at 37°C, 5% $CO_2$. Media was removed and 0.1% (w/v) crystal violet in 20% (v/v) ethanol was added for approximately 7 min. The monolayers were then rinsed with water and the plaques were counted after the plates were dry.

F. Precursor Frequency Determination using Limiting Dilution Analysis:

In vitro stimulation and LDA were performed using the method by Koup et al (32)with modification.

1. Vaccinia Vectors: Vaccinia vectors vac (non-recombinant NYCBH vaccinia) and vv271 which expresses env of the HIV-1 IIIB strain derived from the BH10 molecular clone (designated as IIIB in the Results section) were provided by Therion Biologics. Table 6 summarizes the vac constructs derived from clinical isolates.

2. Feeder cells: PBMC were isolated using Ficoll from freshly drawn heparinized blood from healthy seronegative donors and stimulated for 48 hrs with 5U/ml IL-2 in RPMI with 10% FCS. They were then irradiated with 3500R and resuspended in RPMI containing 15% FCS, 30U/ml IL-2 and 2.5 ul/ml OKT3 at a concentration of either 5 x $10^6$ cells/ml or 2.5 x $10^6$ cells/ml to act as feeders for PBMC derived from infants at very early time points or later time points respectively.

3. Effector cells: PBMC were isolated from freshly drawn heparinized blood from HIV-1-positive infants, quantitated and cryopreserved in 10% DMSO, RPMI, 10% FCS using a cell freezing program (KRYO 10 series) and stored in liquid nitrogen as part of a patient PBMC repository. These were then thawed rapidly, washed and resuspended at 160,000 cells/ml in RPMI containing 15% FCS, 30U/ml IL-2 and 2.5 ul/ml OKT3. One hundred ul of each serial dilution of these cells were then plated into 24 wells of a 96-well U-bottom plate, ranging from 16,000 cells/well to 500 cells/well. PBMC from every time point examined for each patient were stimulated with the same donor cells to lessen interassay variability.

4. *In vitro* non-specific stimulation: One hundred ul of irradiated PBMC feeders were added to each well of effectors and incubated for 5-7 days (until visible cell growth had occurred) at 37°C, 5% $CO_2$. Media were exchanged every fourth day.

5. Target cells: Autologous B-lymphoblastoid cell lines were established from each patient by infection of PBMC with supernatant from the EBV-producing cell line B95.8 in RPMI, 15% FCS, 10% B95.8 supernatant and 2 ug/ml cyclosporin A. Transformed B lymphoblastoid cells were subsequently grown and maintained in RPMI, 15% FCS.

Table 7. Recombinant vaccinia vectors derived from patient env sequences

| *Patient* | *1st time point* | *2nd time point* |
|---|---|---|
| VI-06 | vvAR-1D | ND |
| VI-05 | vvHE-2M | vvHE-6M |
| VI-08 | vvJA-20D | vvJA-6M |
| VI-11 | vvRO-1M | vvRO-3M |

Recombinant vaccinia vectors expressing env genes of the viral isolates cultured from patients VI-06, VI-05, VI-08 and VI-11 were generated through *in vitro* recombination between the env subclones in pAbT4587A and wild type vaccinia as described in the Materials and Methods section.

6. Cytotoxicity Assay: a. Effectors: Effector cells were divided by the number of targets being assayed into 96-well U-bottom plates. RPMI with 15% FCS was added to each well to bring the total volume to 100 ul. b. Targets: 1. Vaccinia-infected targets: Autologous B-LCLs were infected with vv at an MOI of 5:1 for 16 hr at 37°C, 5% $CO_2$ in RPMI, 15% FCS with an initial 60 min incubation at a concentration of 3 x $10^6$ cells/ml with gentle agitation followed by 15hr of incubation at 1 x $10^6$ cells/ml. They were then washed and radiolabeled with 100uCi $Na^{51}Cr$ (NEN)/1 x $10^6$ cells at a concentration of 3 x $10^6$ cells/ml in RPMI, 15% for 1 hr at 37oC with gentle agitation. Following incorporation of label, the targets were washed twice and resuspended in RPMI, 15% FCS at a concentration of 1 x $10^5$ cells/ml. One hundred ul of these were then added to the wells containing effector cells. Twenty four wells were reserved for each target into which only target cells and media were mixed (in the absence of effector cells) and were designated as spontaneous release wells. Fifty ul of each target was pipetted into 6 replicate tubes and were used to measure average maximal chromium release of the targets. The plates containing targets and effectors were centrifuged at 1500 rpm for 3 min. and incubated for 7 hr at 37°C, 5% $CO_2$. 2. Peptide-pulsed targets: Autologous B-LCL were coated with peptide by incubation at 37°C for 1.5 hr with 200 ug/ml peptide in RPMI, 20% FCS and simultaneously radiolabeled with 100 uCi $Na^{51}Cr$/1 x $10^6$ cells at a cell concentration of 3 x $10^6$ cells/ml. The targets were then handled as described above. c. Peptides: Peptides 1923 and 1930 were received from the AIDS Research and Reference Reagent Program (AnaSpec). Peptide V3-1 was prepared at UMMC peptide facility . Lyophylized peptide was resuspended in 10% DMSO in PBS to a concentration of 2 mg/ml. Peptide sequences are as follows: 1923: VPVWKEATTTLFCASDAKAY, 1930: WDQSLKPCVKLTPLCVTLNC and 291: SVEINCTRPNNNTRKSI. d. Assay readout: The plates containing targets and effectors were centrifuged as above at the end of 7 hrs and 100ul of supernatant from each well was collected and radioactively quantitated in a

Beckman gamma counter. e. Calculations: Percent lysis was calculated for each well using the formula 100 x [(test cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] using a MacIntosh Excel Spreedsheet created by Dr. Spyros Kalams, Massachusettes General Hospital. The cut-off for a positive response was above 10-20% lysis depending upon the amount of background within the assay. Simple linear regression analysis was performed by using the least mean squared method. Precursor cell frequency was calculated by solving the equation of the best fit line in which the negative log fraction of the nonresponding wells equaled one (fraction of nonresponding wells = 0.37). Ninety-five percent confidence limits were calculated based upon the true mean value of the dependent variable, PBMC per well. Split-well analysis was also performed and precursor frequencies of CTL recognizing multiple targets were determined using this worksheet. Wells positive for lysis of more than one env-expressing target were considered negative if they were also positive for lysis of wild-type vaccinia virus-expressing targets. The number of negative responses was determined using this criteria for multiple env-expressing target recognition and entered into the spreedsheet to obtain the precursor frequency. The percentage of pCTL cross-reactive with each combination of env-expressing targets was then calculated based upon the precursor frequencies of pCTL able to recognize single env-expressing targets and pCTL able to recognize more than one env-expressing target.

## G. Antibody-Dependent Cellular Cytotoxicity Assay:

ADCC antibody assays were performed according to the method of Koup et al (52)

1. Vaccinia Vectors: The recombinant vaccinia vectors used were those described above.

2. Plasma: Plasma derived from HIV-1 positive infants at time points ranging from 2 months to 2 years of age was heat-inactivated at 56°C for 45 minutes. Plasma was then serially diluted with RPMI, 15% FCS using half log dilutions from $10^{-2}$ to $10^{-5}$. Plasma from HIV-1-infected individuals with hemophilia was pooled, heat-inactivated and used as a positive control.

3. Effectors: PBMC were isolated from freshly drawn heparinized blood from healthy HIV-1 seronegative donors and resuspended to a concentration of 2.5 x $10^6$ cells/well.

4. Targets: A B-LCL line designated MC-LCL was used in all assays at a concentration of 1 x $10^5$ cells/well. These were infected with vaccinia and labeled with $Na^{51}Cr$, as previously described.

5. ADCC assay: The ADCC assay was a modification of that reported by Koup et al . Twenty ul of diluted plasma was aliquotted in triplicate into wells of a 96-well U-bottom plate for each plasma sample. A triplicate set of wells designated as "spontaneous release" received 20 ul of plasma at the lowest dilution. Ninety ul of targets were added to each well and incubated for 30 min at 37°C, 5% $CO_2$. Following this incubation, 90 ul of effectors were added to each well except for those designated as spontaneous release wells. The plates were centrifuged at 1500 rpm for 3 min and incubated at 37°C, 5% $CO_2$ for 8 hr. An additional 96-well plate was also set up in triplicate to measure chromium release in the absence of plasma in which 20 ul of RPMI, 15% FCS was added in place of plasma with the same effector and target cell input. Forty five ul of each of the target cells were collected into 6 tubes to measure average maximal chromium release. After 8 hr., the plates were centrifuged as above and 100 ul of supernatant were collected and counted in a Beckman gamma counter.

6. Calculations: Percent lysis for each plasma dilution was determined by the formula: 100 x [(test cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)].

Percent ADCC cytotoxicity = percent lysis of targets with plasma - percent lysis of targets in the absence of plasma. The antibody titer of the plasma was determined by calculating the mean and standard deviation of ADCC lysis of wild type vac infected targets at each plasma dilution with each plasma sample tested. From these values, a cut-off value of two standard deviations above the mean was established for each plasma dilution. The antibody titer against env-expressing targets was then defined as the highest plasma dilution which still gave an ADCC lysis value above the cut-off for that dilution.

# CHAPTER III. RESULTS

## A. Diversity of patient proviral DNA sequences from IIIB

Past studies had measured infant immune responses against HIV-1 with a IIIB based vector, 271, which was derived from IIIB env. Genetic differences between IIIB env and the autologous HIV-1 env replicating in vivo may explain the lack of detection of CTL within early infancy. Therefore, the amount of diversity from IIIB was examined in each of the patient's proviral DNA through heteroduplex formation with pAbT4603. The PCR product analyzed covers hypervariable regions V1-V3 within the env gene. HIV-$1_{MN}$ and HIV-$1_{IIIB}$ differ by 8% within this region as determined using best fit analysis in GCG in Genetics Computer Froup's Wisconsin Sequence Analysis Package version 8.0.1 using the default program parameters. Figure 1 shows that the distance of migration of the heteroduplexes formed between the patient proviral DNA and IIIB env are close to that of MN env and IIIB env. This suggests that the amount of diversity of the patient isolate sequences from IIIB within env is at least 8%. Furthermore, a comparison of the relative mobilities from the early and later time points from each individual probed with IIIB shows little difference. Relative mobilities were obtained for the heteroduplexes by calculating the average distance of migration from the wells of the gel of the heteroduplex bands divided by the distance of migration of the homoduplex bands. Using the formula by Delwart, et al.(2), DNA distance = -ln[(mobility-0.045)/1.14]/13.6, derived by plotting relative heteroduplex mobility against percent DNA distance, the diversity within this region ranged from 4.8 - 5.4% among the patient isolates (Figure 2).

Analysis of the heteroduplexes which formed between IIIB env and patient proviral env demonstrate differences in patient env heterogeneity at the two time points examined

(figure 1). A greater extent of heterogeneity was observed within proviral env sequences at 1 day of age than at 6 months of age of patient VI-06. This was represented by the formation of a multiple heteroduplexes. These heteroduplexes migrated closely suggesting that they were closely related to one another. There appeared to be only one predominant env sequence at 6 months, represented by the formation of two heteroduplexes. Proviral env from patient VI-05 developed greater heterogeneity by 6 months of age. At 2 months of age there appeared to be only one predominant env sequence. Env sequences of patient VI-08 did not become more heterogeneous from 20 days of age to 6 months. At each time point there appeared to be only one predominant sequence. However, comparison of these two time points demonstrated an env sequence which is more closely related to IIIB at 20 days of age. Proviral sequences of patient VI-11 appeared to be identical at each time point although clones derived from these samples differed.

Figure 1. Heteroduplexes formed between patient proviral DNA and IIIB env

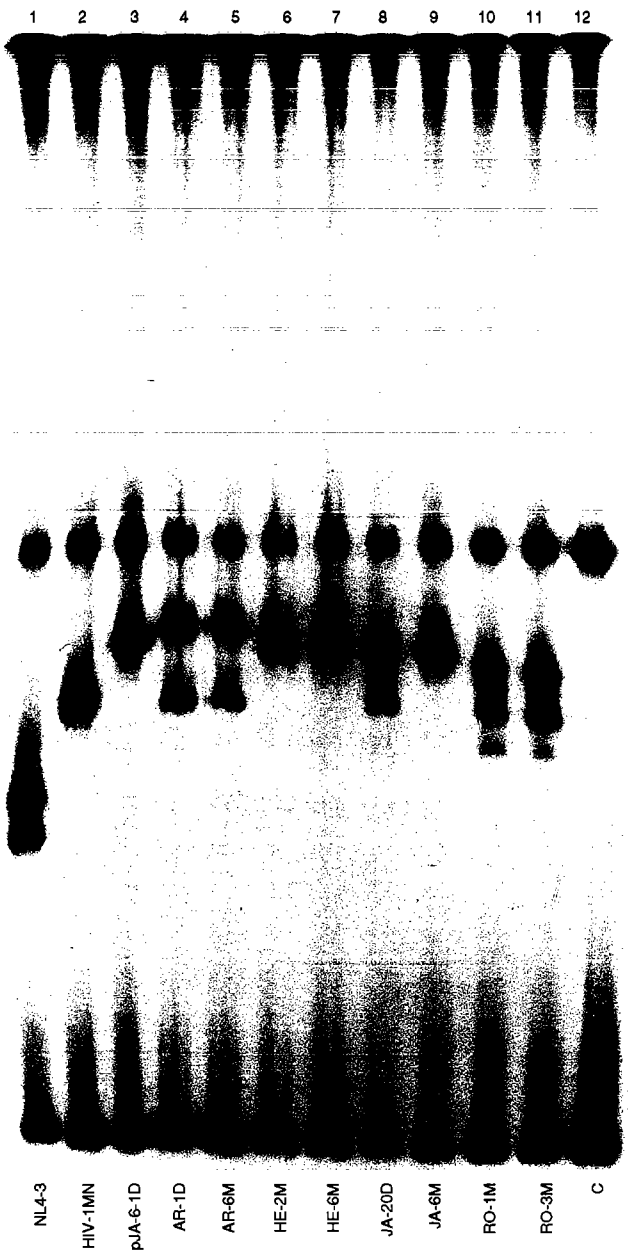
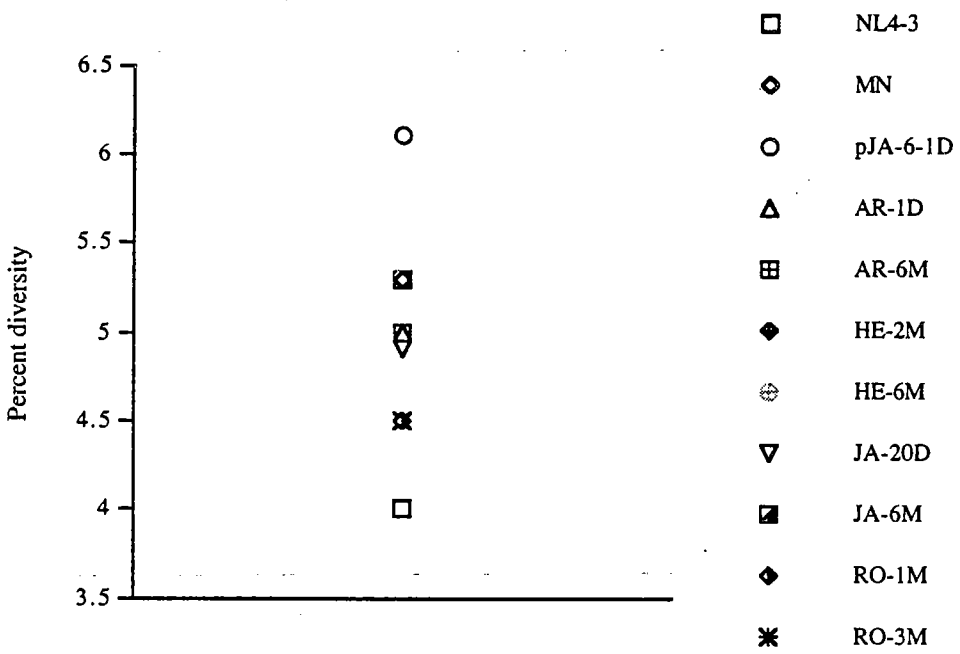


Figure 1. Heteroduplexes formed between patient proviral DNA and IIIB env

Env fragments were amplified by PCR from genomic DNA extracted from PBMC infected with patients HIV-1 isolates and from a plasmid containing BH10 (IIIB) env (pAbT4603). The PCR fragment is a 946 bp product which contains sequences within env which span V1 to V3. The PCR product of pAbT4603 was internally radiolabeled during the PCR and combined with the PCR products of the patient's proviral templates to form heteroduplexes as described in the Materials and Methods section. Lanes 1-3 are clones of known sequence probed with pAbT4603. Lane 1 is a PCR product of pNL4-3 which contains the LAV strain of HIV-1 (clade B). Lane 2 is a PCR product of env of the MN strain of HIV-1 (the prototypical clade B strain of HIV-1) using plasmid pT2020 as template and lane 3 is a PCR product of env from an env clone derived from patient VI-08 (pJA-6-1D). Lane 4-11 are PCR products amplified from patient proviral DNA (VI-06 is AR-1D and AR-6M, VI-05 is HE-2M and HE-6M, VI-08 is JA-20D and JA-6M and VI-11 is RO-1M and RO-3M). Lane 12 is a negative control of probe alone.

Figure 2. Percent genetic distance of patient proviral DNA from IIIB




Relative mobilities of the heteroduplexes depicted in figure 4 were obtained by measuring the average migration of the heteroduplexes from the wells of the gel divided by the distance of migration of the homoduplexes. Percent diversity was determined using the formula DNA distance = -ln[(mobility- 0.045)/1.14]/13.6 (2).

B. Recombinant vaccinia virus construction

1. Amplification and cloning of the env gene using PCR from infant isolates: The env gene was successfully amplified and cloned using primers which annealed upstream and downstream of the gene (figure 3). These PCR products were subsequently ligated into the pCR3 backbone from which 60 env clones were derived and categorized (Table 8) based upon restriction digest patterns. One env clone which was predominant was selected for each patient and subcloned into pAbT4587A. The selected clones are presented in table 8. Each env gene was then subcloned into pAbT4587A and used to generate recombinant vaccinia virus. The env gene from pCAR-6M could not be subcloned because of lack of stability of this gene in pAbT4587A. Therefore, a total of 7 env subclones were generated. Clones and subclones of patient env sequences were analyzed through heteroduplex formation to ensure identity of the env genes cloned. The derivation of all env genes subcloned into pAbT4587A was verified using identical env genes ligated into the pCR3 backbone as probe (figure 4). Absence of contaminating env sequences was shown by the lack of heteroduplex formation between these env fragments. Because pNL4-3 was used frequently as a positive PCR control, it was also used as a probe to make sure that none of the cloned env sequences were in fact pNL4-3. The formation of heteroduplexes of varying mobility between pNL4-3 and these env fragments further verified lack of contamination and demonstrated that each of the env clones was unique to one another.

2. Expression analysis of subcloned env genes: Patient env genes in pAbT4587A were analyzed for their ability to encode env protein. This was accomplished through transfection of the env subclones into vaccinia-infected cells. Expression of the env gene is driven by the 40K vaccinia promoter which is activated by the presence of wild type vaccinia within the transfected cell. Incubation of these cells with HIV-1 env-specific rabbit sera followed by a phosphatase-labeled goat anti-rabbit second antibody

demonstrated the presence of env protein by the appearance of blue-stained cells (figure 5). Cell surface membrane staining was the predominant pattern. All of the subcloned env's demonstrated the ability to make env protein.

3. Expression analysis of recombinant vaccinia vectors derived from patient env genes: Recombinant vaccinia virus was derived from the seven env clones described above. Prior to amplification of these vectors, their ability to encode env protein was examined by immunoprecipitation (figure 6). All of the vectors were shown to encode for gp160 and its cleavage product, gp120 (gp41, a second cleavage product migrated beyond the detectable limit of the gel).

## C. Precursor Frequency of CTL recognizing heterologous and autologous env-expressing targets of patients VI-06, VI-05, VI-08 and VI-11

CTL precursor frequencies were measured in all of the patients at ages at which we had been unable to detect IIIB-env-specific CTL responses. Although IIIB-env-specific CTL were not present, CTL precursors from 3 out of 4 of these patients recognized env from autologous viral isolates at frequencies within a range (39-130 pCTL/$10^6$ PBMC) (table 10) of those reported against laboratory strains of HIV-1 in older children (67). Patient VI-06, a patient who had previously been shown to have pCTL against the IIIB gag gene product within cord blood (68), possessed low numbers of pCTL in cord blood against the env gene product cloned from virus present at 1 day of age (figure 7A). Although the frequency of pCTL against autologous env protein is not statistically higher than background, previous detection of pCTL in cord blood from this patient supports the possibility that the higher frequency of pCTL detected against this env product is real. CTL precursors from patient VI-08 at 6 months of age were highest against the env gene product cloned from virus present at 20 days of age (figure 9A). A lower frequency of pCTL was

detected from this patient at this time point against the env gene product cloned from virus present at 6 months of age. This lower frequency was not statistically higher than background but was high enough to suggest possible recognition of this env gene product as well. At 3 and 7 months of age, pCTL from patient VI-11 recognized both env gene products cloned from virus present at 1 month and 3 months of age (figure 10A and B). No pCTL were detected in the cord blood of patient VI-05 against either autologous env gene products or IIIB (figure 8A).

All four patients demonstrated pCTL able to recognize IIIB and autologous env gene products by at least 12 months of age. Patient VI-06 demonstrated group-specific CTL responses as early as 6 months of age (figure 7B). Group-specific CTL responses at this age are considered to be unusually early as IIIB responsiveness, measured previously in our laboratory and in this study, was not detected in the other 3 patients until approximately 12 months of age. CTL precursors from patient VI-05 at 12 months of age recognized both the IIIB env and the env gene product cloned from virus present at 2 months of age (figure 8B). Curiously, pCTL from this patient at 12 months of age were not able to recognize the env gene product cloned from virus present at 6 months of age. By 19 months of age, pCTL from patient VI-08 recognized the IIIB env and the env gene products cloned from virus present at 20 days and 6 months of age (figure 9B). In comparison to the level of recognition at 6 months of age of the env gene product cloned from virus present at 6 months of age, the frequency of pCTL recognizing this env protein by 19 months of age was significantly higher than background. By 12 months of age, patient VI-11 possessed pCTL able to recognize the IIIB env gene product as well as both autologous env gene products (figure 10C).

Figure 3. Env PCR products derived from patient provirus

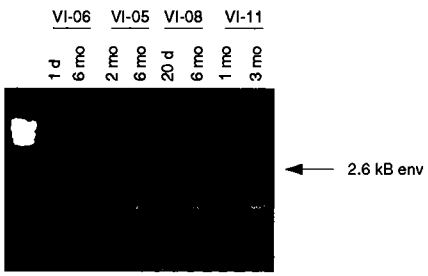


Figure 3. Env PCR products derived from patient provirus.

Genomic DNA from PBMC infected with viral supernatant from HIV-1 isolates from patients VI-06, VI-05, VI-08 and VI-11 described in table 2 was used as template to amplify the entire coding region of env by PCR as described in the Materials and Methods section. Molecular weight standards are in lane 1. Lanes 2-9 are PCR products derived from patient proviral DNA using primers MNA and MN13 described in table 4.

Table 8: Grouping of env clones of patient isolates within pCR3 based upon restriction digests with StuI, AvaI, BamHI, KpnI, BglI and HindIII:

| Group | Patient, age | Correct* | Reverse* | Percent of clones‡ |
|---|---|---|---|---|
| 06-1D-A | VI-06, 1d | 1-3 | 1-2 | 22 |
| 06-1D-B | | | 1-9 | 11 |
| 06-1D-C | | 1-13,1-15,1-20,1-18 | 1-11,1-12 | 56 |
| 06-1D-D | | 1-18 | | 11 |
| 06-6M-A | VI-06, 6 mo | 2-1,2-7,2-8,2-9 | 2-2,2-3,2-4,2-10 | 89 |
| 06-6M-B | | | 2-5 | 11 |
| 05-2M-A | VI-05, 2 mo | 3-4,3-5,3-7,3-8,3-10 | 3-2 | 86 |
| 05-2M-B | | 3-3 | | 14 |
| 05-6M-A | VI-05, 6 mo | 4-2,4-5,4-8,4-9 | 4-4,4-6 | 100 |
| 08-20D-A | VI-08, 20d | | 5-4 | 14 |
| 08-20D-B | | | 5-6 | 14 |
| 08-20D-C | | 5-2,5-3,5-8,5-9,5-10 | | 72 |
| 08-6M-A | VI-08, 6 mo | 6-1 | | 12 |
| 08-6M-B | | 6-2,6-3,6-4,6-7,6-8,6-9,6-10 | | 88 |
| 11-1M-A | VI-11, 1 mo | | 7-1 | 11 |
| 11-1M-B | | 7-10,7-11,7-19 | 7-2,7-5,7-7,7-16 | 78 |
| 11-1M-C | | | 7-12 | 11 |
| 11-3M-A | VI-11, 3 mo | | 8-3 | 17 |
| 11-3M-B | | 8-4,8-11,8-14,8-16, | | 83 |

Env genes amplified by PCR from provirus of patients VI-06, VI-05, VI-08 and VI-11 at the time points indicated in table 2 were ligated into pCR3 (InVitrogen) and digested with StuI, AvaI, BamHI, KpnI, BglI and HindIII. One to four distinct patterns of restriction digests were obtained from each set of clones derived from virus from each patient at a single time point which were designated as groups A, B, C and D. Individual env clones were categorized into each group on the basis of their restriction digest patterns. 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.

* Based upon orientation of gene relative to control of CMV promoter of pCR3

‡ Percent of clones from the total number derived from one patient at one time point which fell within a particular group

Table 9: Env clones selected to be subcloned into pAbT4587A:

| *Patient, age* | *clone* | *new designation* |
|---|---|---|
| VI-06, 1 day | p1-13 | pCAR-1D |
| VI-06, 6 months | p2-1 | pCAR-6M |
| VI-05, 2 months | p3-4 | pCHE-2M |
| VI-05, 6 months | p4-2 | pCHE-6M |
| VI-08, 20 days | p5-2 | pCJA-20D |
| VI-08, 6 months | p6-2 | pCJA-6M |
| VI-11, 1 month | p7-11 | pCRO-1M |
| VI-11, 3 months | p8-4 | pCRO-3M |

Predominant env clones were chosen from the clones presented in table 7 as described in the legend for table 7 and in the Materials and Methods section. The env gene from these clones were then subcloned into pAbT4587A as described in the Materials and Methods section.

Figure 4. Heteroduplex analysis of cloned and subcloned env genes

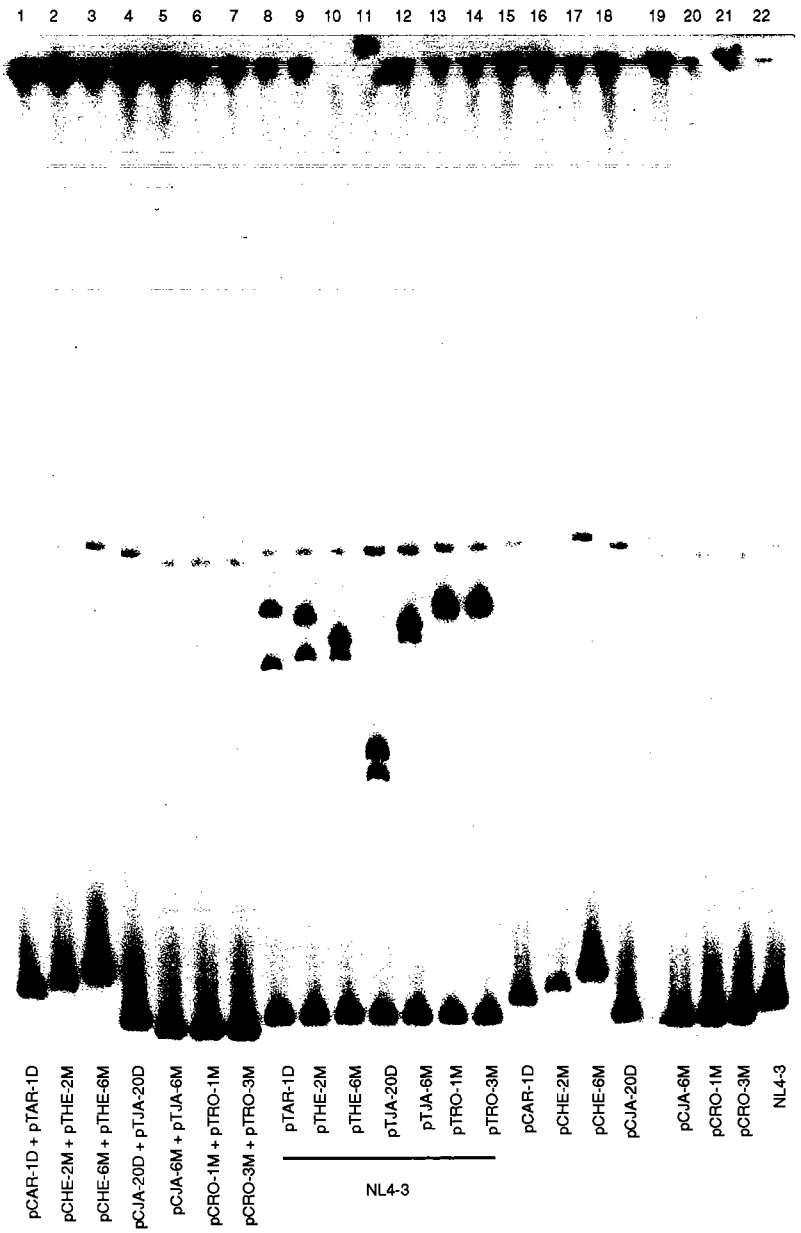


Figure 4. Heteroduplex analysis of cloned and subcloned env genes.

Env fragments were amplified by PCR from env clones within the pCR3 and pAbT4587A backbones described in tables 7 and 8 and from pNL4-3. The PCR fragment is a 946 bp product which contains sequences within env which span V1 to V3. 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 the Materials and Methods section. 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 env genes probed with pNL4-3. Lanes 15-22 are negative controls containing radiolabeled PCR products alone.

Figure 5. Cell surface staining of cells transfected with env plasmids

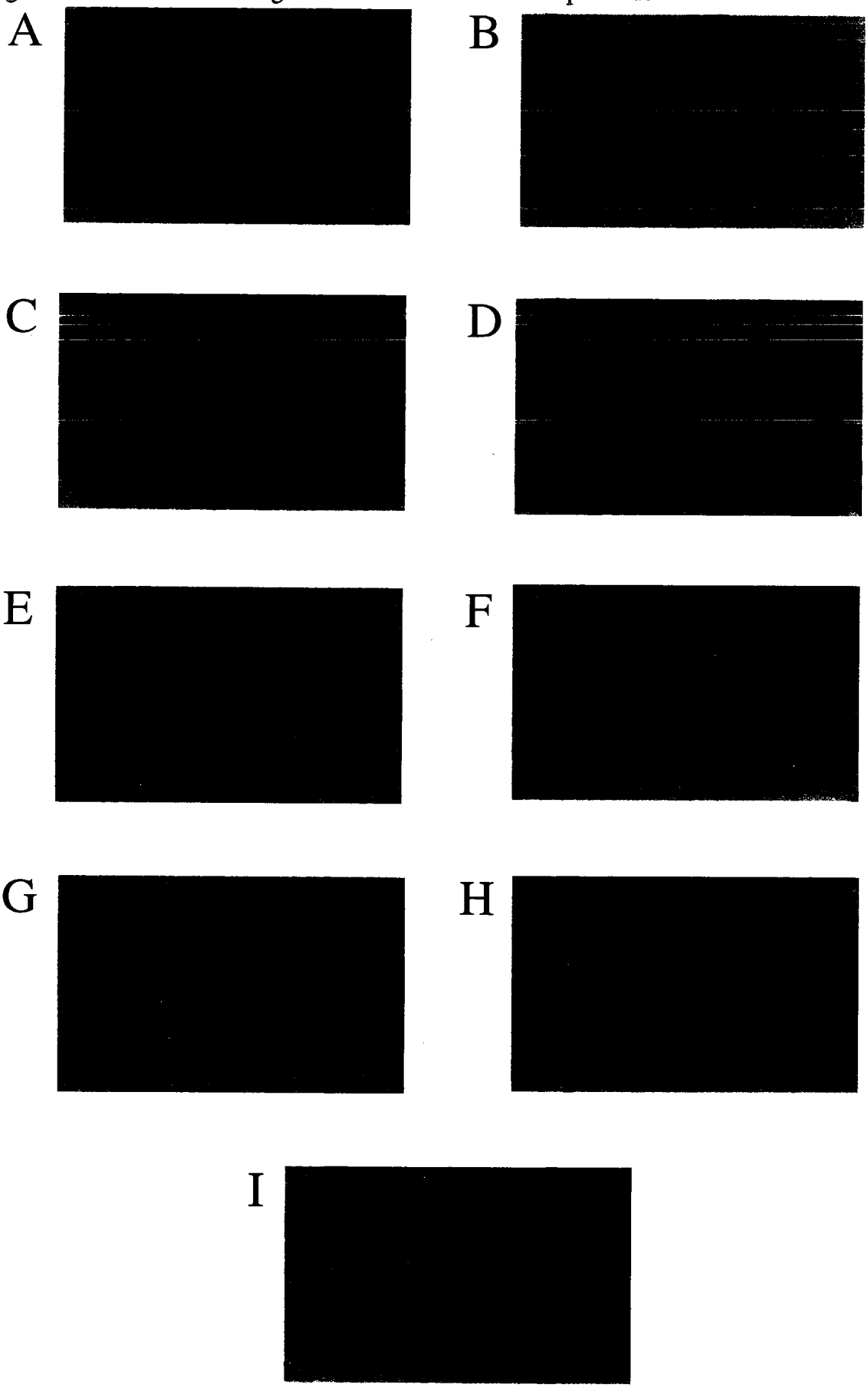


Figure 5. Cell surface staining of cells transfected with env plasmids.

RK-13 cells were infected with wild-type vaccinia virus and transfected with env gene-containing clones in the pAbT4587A or, in the case of pAbT4603, pAbT4587 backbone as described in the Materials and Methods section. These cells were then incubated with sera from HIV-1 immunized rabbits. Env expression was detected by purple staining resulting from incubation of these cells with a secondary goat-anti-rabbit antibody conjugated to alkaline phosphatase and treated with appropriate substrate. Plasmids used in transfection: A, mock. B, pAbT4603. C, pTAR-1D. D, pTHE-2M. E, pTHE-6M. F, pTJA-20D. G, pTJA-6M. H, pTRO-1M and I, pTRO-3M.

Figure 6. Immunoprecipitation of CV-1 cell lysates infected with env-recombinant vaccinia vectors

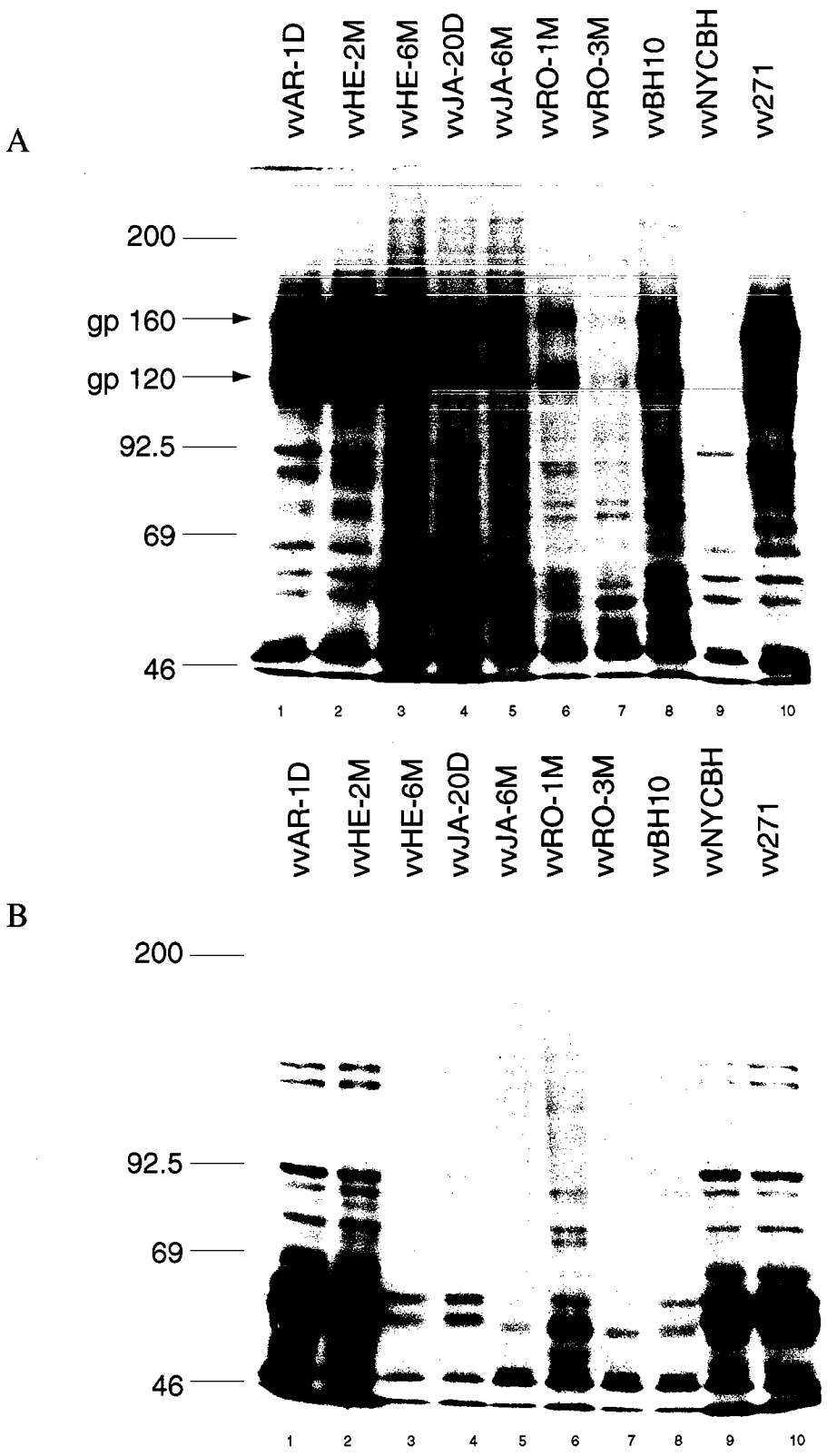


Figure 6. Immunoprecipitation of CV-1 cell lysates infected with env-recombinant vaccinia vectors.

CV-1 cells were infected with crude viral preparations of env-recombinant vaccinia viruses. These cells were lysed with detergent and the lysates were incubated with either pooled sera from HIV-1 infected individuals with hemophilia or pooled sera from HIV-1 negative individuals. Immune complexes were captured by protein A-sepharose and analyzed as described in the Materials and Methods section. A: Cell lysates immunoprecipitated with pooled sera from HIV-1 infected individuals with hemophilia. Lanes 1-7 are vaccinia recombinants of patient-derived env genes, lane 8 is a recombinant of env derived from pAbT4603 generated as a positive control for vaccinia vector construction, lane 9 is wild type NYCBH vaccinia and lane 10 is an env recombinant vaccinia vector from Therion Biologics used as a positive control for immunoprecipitaton. B: Lanes are identical to those in A but preciptated with pooled sera from HIV-1 negative individuals.

Table 10. CTL precursor frequencies of patients VI-05, VI-06, VI-08 and VI-11 over time against autologous env gene products and IIIB env

| | | CB | 3 MO | 6 MO | 7 MO | 12 MO | 19 MO |
|---|---|---|---|---|---|---|---|
| Patient | vv | | | | | | |
| VI-06 | vac | 34 (23-50) | | 0.5 (0.03-8) | | | |
| | IIIB | 13 (7-23) | | 153 (118-200) | | | |
| | vvAR-1D | 64 (47-87) | | 197 (151-257) | | | |
| VI-05 | vac | 18(11-30) | | | | 65 (48-89) | |
| | IIIB | 25 (16-39) | | | | 219 (169-284) | |
| | vvHE-2M | 31 (21-47) | | | | 129 (99-168) | |
| | vvHE-6M | * | | | | 55 (40-76) | |
| VI-08 | vac | | | 50 (36-70) | | | 19 (12-32) |
| | IIIB | | | 50 (35-70) | | | 201 (154-262) |
| | vvJA-20D | | | 130 (99-170) | | | 81 (60-108) |
| | vvJA-6M | | | 80 (60-107) | | | 175 (135-229) |
| VI-11 | vac | | 8 (4-17) | | 3 (0.8-10) | 25 (16-39) | |
| | IIIB | | 23 (14-36) | | 4 (1-11) | 62 (45-85) | |
| | vvRO-1M | | 136 (105-177) | | 42 (29-60) | 176 (136-228) | |
| | vvRO-3M | | 138 (107-180) | | 70 (52-94) | 74 (55-99) | |

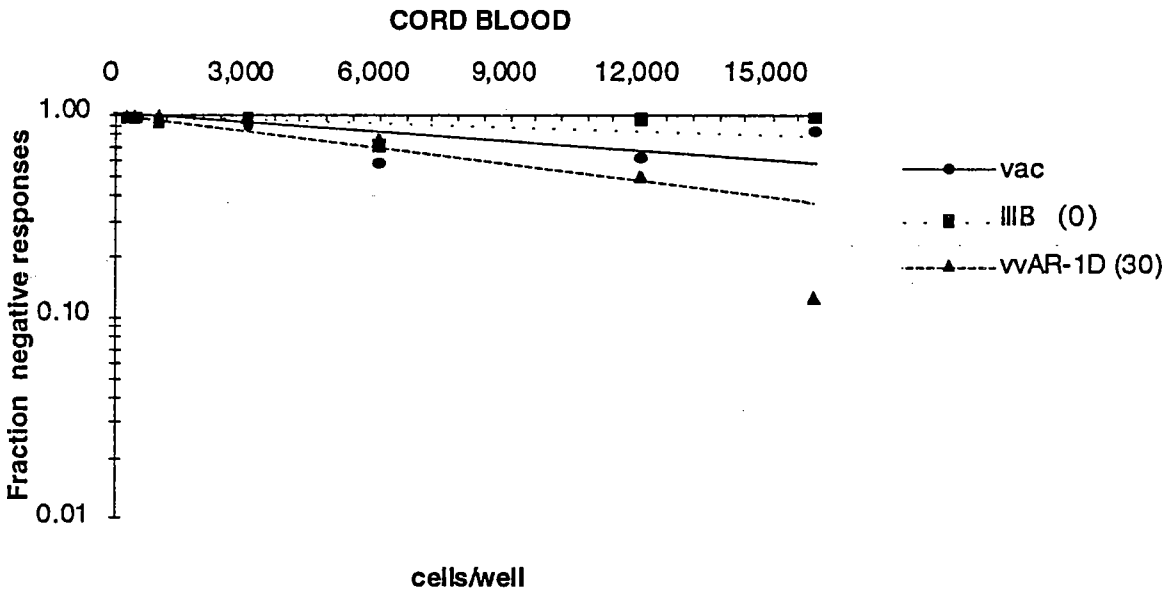
Table 10. CTL precursor frequencies of patients VI-05, VI-06, VI-08 and VI-11 over time against autologous env gene products and IIIB env.

Precursor frequencies of CTL were measured by LDA at the time points indicated as described in materials and methods. Vaccinia vectors used as targets were wild type vaccinia: vac, IIIB env expressing vaccinia: IIIB, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-06 at 1 day of age: vvAR-1D, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-05 at 2 months of age: vvHE-2M, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-05 at 6 months of age: vvHE-6M, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-08 at 20 days of age: vvJA-20D, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-08 at 6 months of age: vvJA-6M, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-11 at 1 month of age: vvRO-1M, and env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-11 at 3 months of age: vvRO-3M. Numbers in parentheses are 95% confidence intervals for each frequency value obtained using an MacIntosh Excel Spreedsheet kindly provided by Dr. Spyros Kalams, Massachusetts General Hospital.

* Spontaneous release for this target was too high to determine a precursor frequency value

Figure 7. Quantitation of HIV-1 env-specific CTL precursors in patient VI-06 in cord blood (panel A) and 6 months of age (panel B)

A.




B.

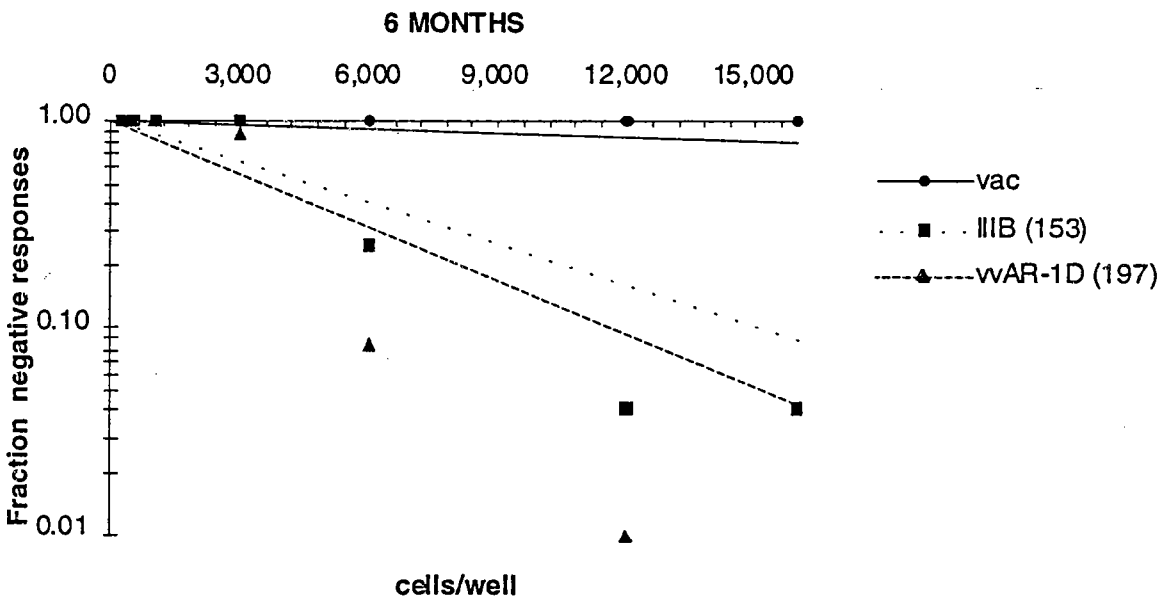
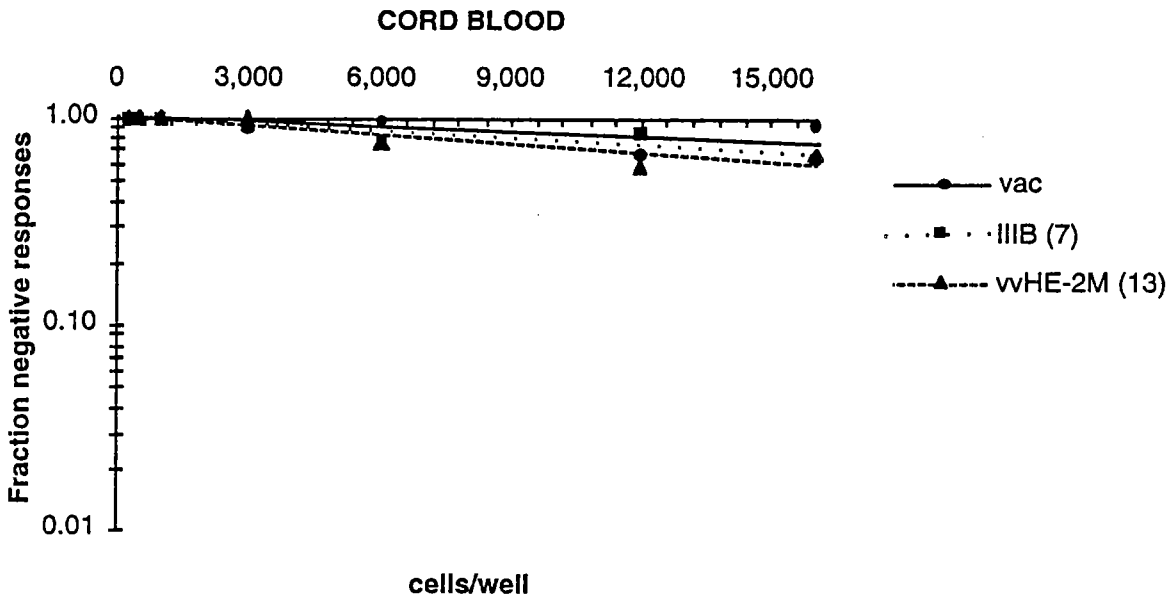


Figure 7. Quantitation of HIV-1 env-specific CTL precursors in patient VI-06 in cord blood (panel A) and 6 months of age (panel B).

CTL precursors were quantitated using limiting dilution assays followed by in vitro stimulation of PBMC from CB and 6 months of age. HIV-1 specific frequencies were calculated as described in the Materials and Methods section. CTL precursor frequencies were measured against wild type vaccinia: vac, IIIB env expressing recombinant vaccinia: IIIB and env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-06 at 1 day of age: vvAR-1D. Numbers in parantheses are pCTL values with vac background subtracted. Spontaneous release was < 30% for all targets shown. The cut-off for a positive response was 20% for the two assays.

Figure 8. Quantitation of HIV-1 env-specific CTL precursors in patient VI-05 in cord blood (panel A) and 12 months of age (panel B).

A.




B.

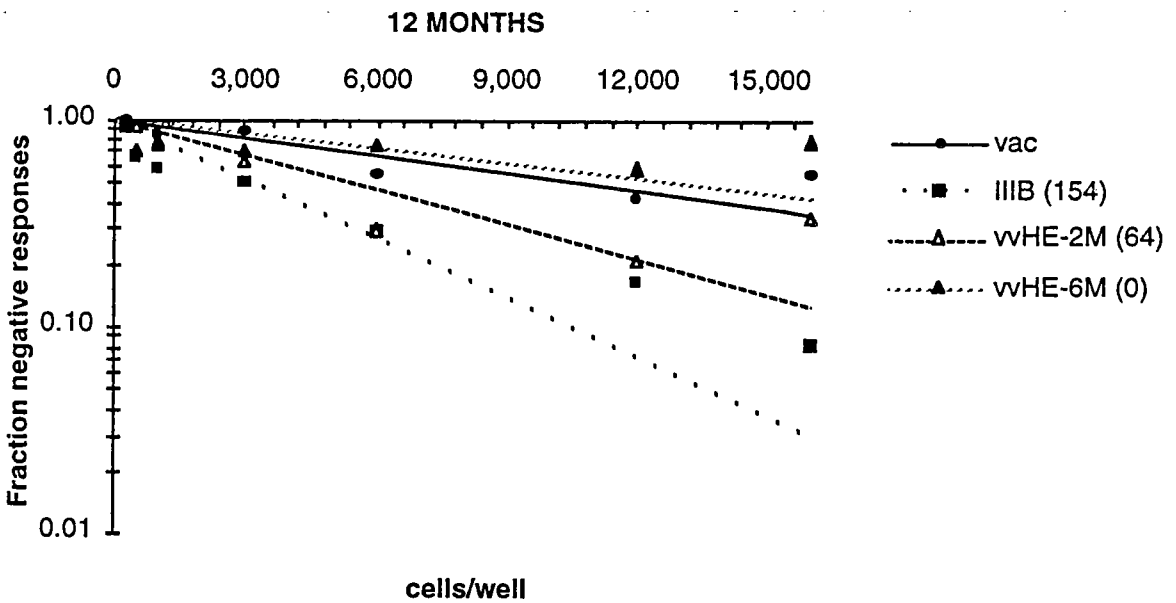
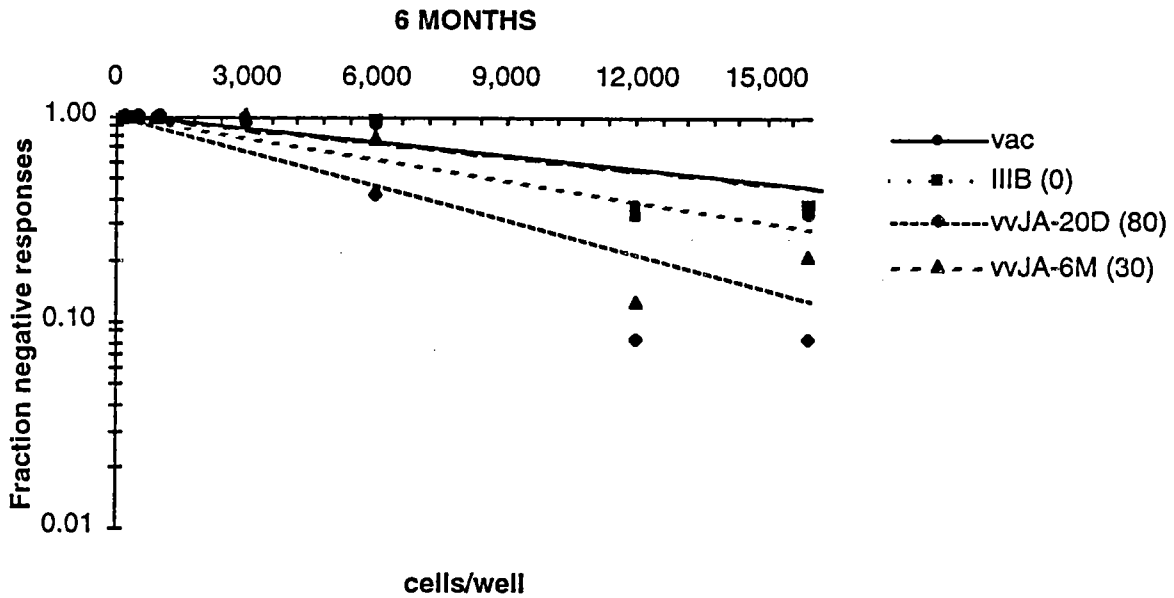


Figure 8. Quantitation of HIV-1 env-specific CTL precursors in patient VI-05 in cord blood (panel A) and 12 months of age (panel B).

CTL precursors were quantitated using limiting dilution assays followed by in vitro stimulation of PBMC from CB and 12 months of age. HIV-1 specific frequencies were calculated as described in the Materials and Methods section. CTL precursor frequencies were measured against wild type vaccinia: vac, IIIB env expressing recombinant vaccinia: IIIB, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-05 at 2 months of age: vvHE-2M, and env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-05 at 6 months of age: vvHE-6M. Numbers in parantheses are pCTL values with vac background subtracted. Spontaneous release was < 35% for all targets shown except vvHE-2M in panel B which was 41%. The cut-off for a positive response was 10% for the assay depicted in panel A and 20% for the assay in panel B.

Figure 9. Quantitation of HIV-1 env-specific CTL precursors in patient VI-08 at 6 months (panel A) and 19 months of age (panel B).

A.




B.

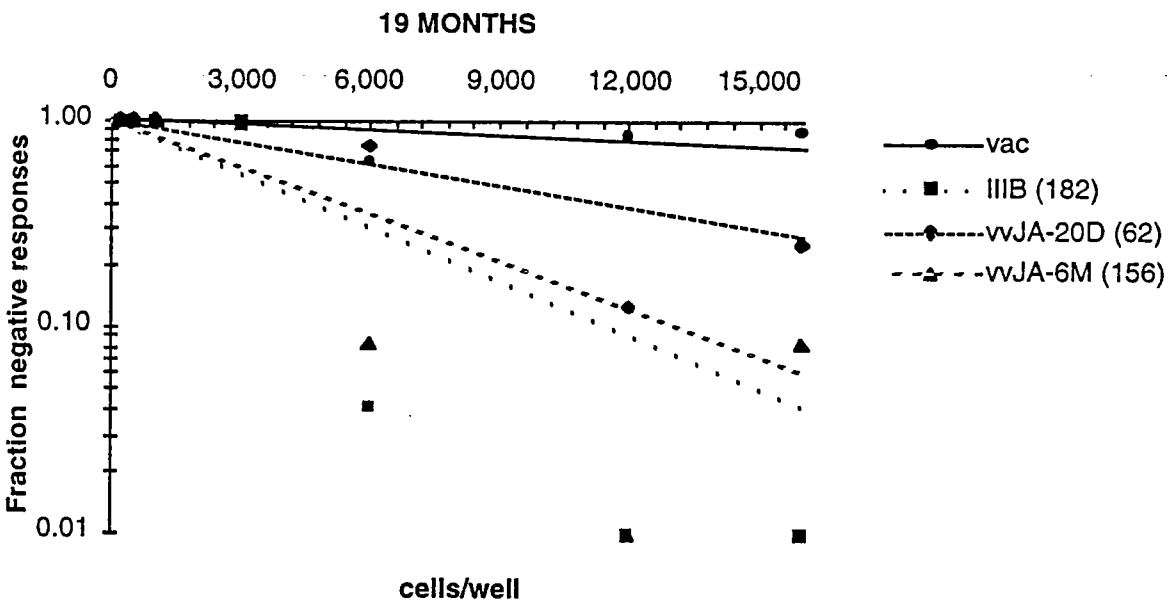
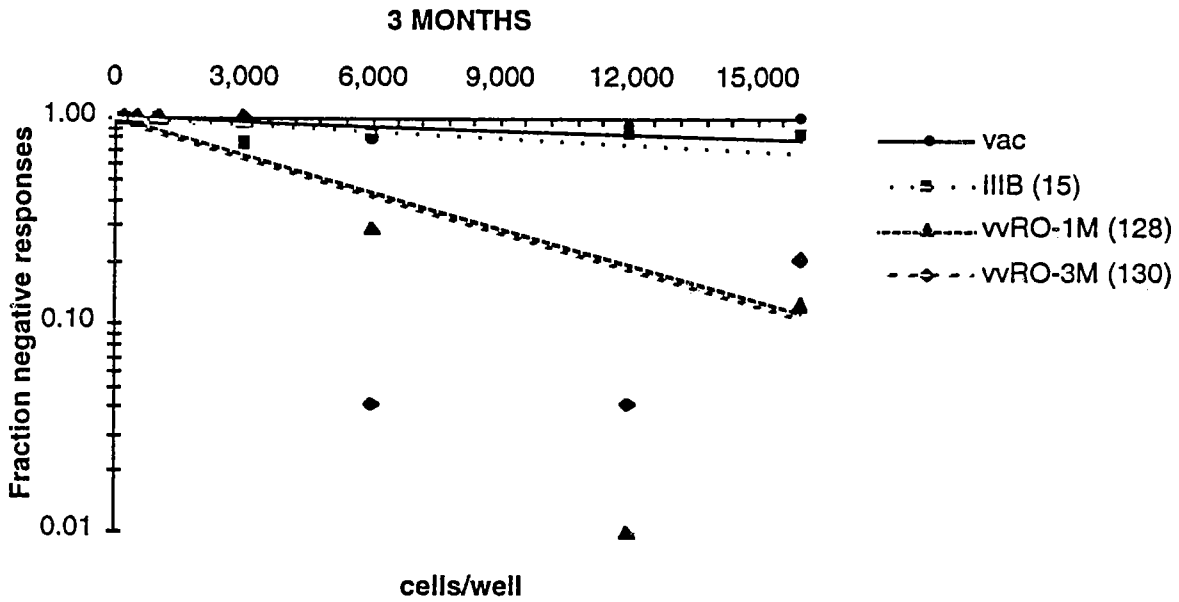


Figure 9. Quantitation of HIV-1 env-specific CTL precursors in patient VI-08 at 6 months (panel A) and 19 months of age (panel B).

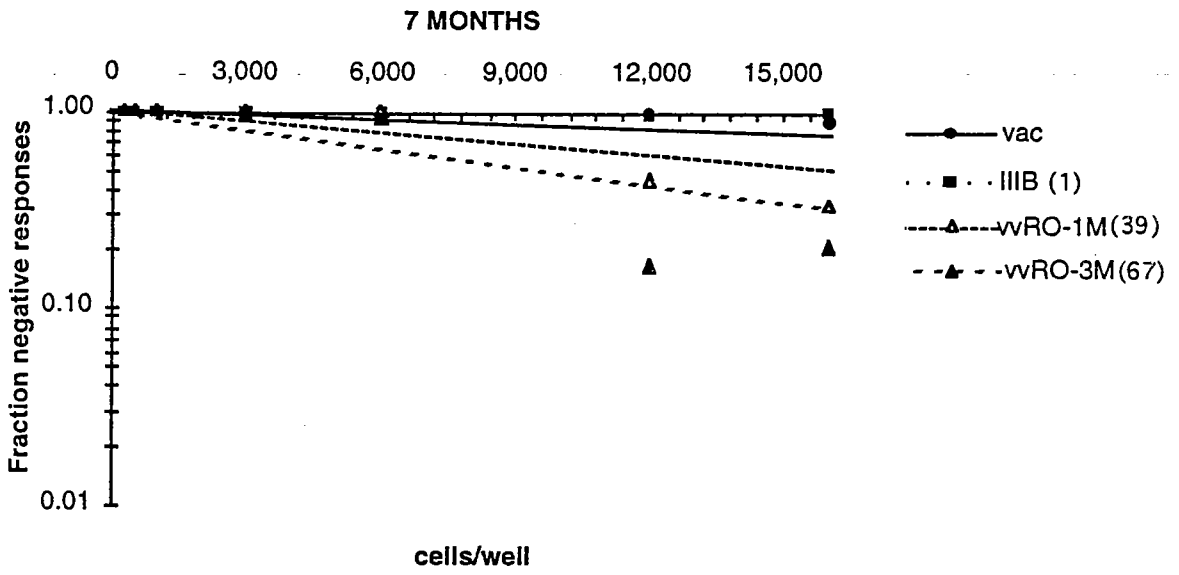
CTL precursors were quantitated using limiting dilution assays followed by in vitro stimulation of PBMC from 6 months and 19 months of age. HIV-1 specific frequencies were calculated as described in the Materials and Methods section. CTL precursor frequencies were measured against wild type vaccinia: vac, IIIB env expressing recombinant vaccinia: IIIB, env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-08 at 20 days of age: vvJA-20D, and env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-08 at 6 months of age: vvJA-6M. Numbers in parantheses are pCTL values with vac background subtracted. Spontaneous release was <33% for all targets shown. The cut-off for a positive response was 30% for both assays.

Figure 10. Quantitation of HIV-1 env-specific CTL precursors in patient VI-11 at 3 months of age (panel A), 7 months of age (panel B) and 12 months of age (panel C).

A.




B.



C.

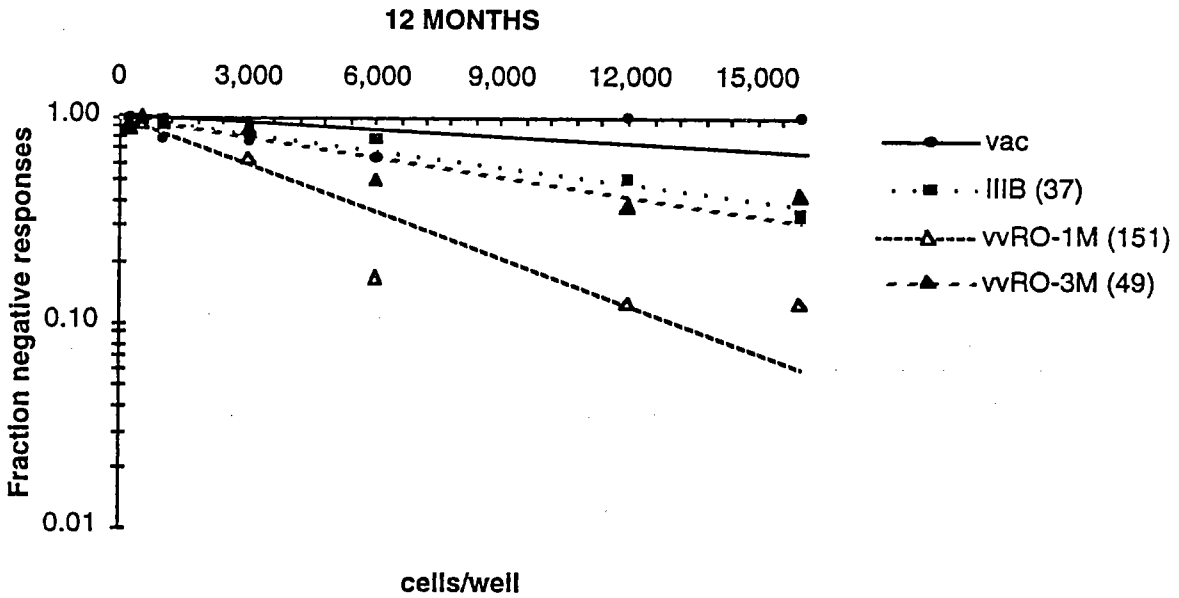

12 MONTHS
0
3,000
6,000
9,000
12,000
15,000
1.00
0.10
0.01
Fraction negative responses
cells/well
vac
IIIB (37)
vvRO-1M (151)
vvRO-3M (49)

Figure 10. Quantitation of HIV-1 env-specific CTL precursors in patient VI-11 at 3 months of age (panel A), 7 months of age (panel B) and 12 months of age (panel C).

CTL precursors were quantitated using limiting dilution assays followed by in vitro stimulation of PBMC from 3 months, 7 months and 12 months of age. HIV-1 specific frequencies were calculated as described in the Materials and Methods section. CTL precursor frequencies were measured against wild type vaccinia: vac, IIIB env expressing recombinant vaccinia: IIIB env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-11 at 1 month of age: vvRO-1M, and env expressing vaccinia derived from an HIV-1 viral isolate from patient VI-11 at 3 months of age: vvRO-3M. Numbers in parantheses are pCTL values with vac background subtracted. Spontaneous release was <35% for all targets shown except for targets expressing vvRO-3M in panel C which was 42%. The cut-off for a positive response was 10% for both assays depicted in panel A and B and 15% for the assay depicted in panel C.

D. Quantitation of cross-reactive and singly reactive pCTL by split-well analysis:

The unique group-specificity of the CTL response of patient VI-06 at six months of age was derived from a pool of cross-reactive pCTL (93% of all the env-specific pCTL detected), rather than two separate pools of pCTL in which one was autologous env-specific and one IIIB-specific. This is in direct contrast to that seen with the same patient in the PBMC of cord blood in which there were only detectable pCTL to autologous env sequences. This suggests that the pCTL measured at 6 months of age were not derived from those detected in cord blood but arose from a separate pool which expanded in vivo between 0 and 6 months. The relationship between the detectable env-specific pCTL measured in these assays is depicted diagramatically in figure 11.

The pCTL detected within the PBMC of patient VI-05 at 12 months of age against env from the patient's 2 month isolate and IIIB env were derived from three different pools. The majority of the pCTL against the 2 month env were derived from a pool cross-reactive with IIIB (41% of all env pCTL), whereas only 14% were specific only for the autologous env. On the other hand, the IIIB-specific pCTL were equally represented by a pool cross-reactive with autologous (41%) and a pool recognizing only IIIB (45%) (figure 12).

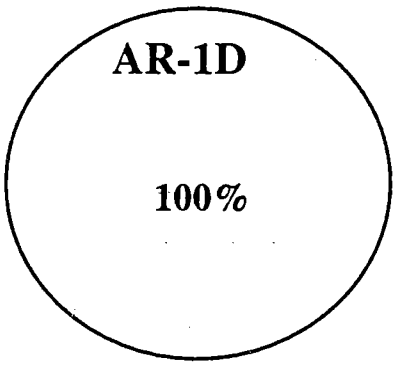
The pCTL detected in patient VI-08 at 6 months of age were only specific for the patient's autologous env from his 20 day isolate. Yet, at 19 months, the pCTL measured against this same env were derived from three pools of cross-reactive pCTL and therefore were probably not from this previous pool. pCTL cross-reactive with all three env's (autologous from 20 day and 6 month isolates and IIIB) were the most highly represented within the total pCTL reactive to the 20 day autologous env (47% of all pCTL against env measured) (figure 13). Although there were no detectable pCTL against autologous env from the sixth month isolate at 6 months of age, by 19 months pCTL reactive to this env

could be measured and these were also derived from cross-reactive pools of pCTL only. The same was true of pCTL responsive to IIIB env at this time point.

At 3 months of age, the pCTL recognizing both autologous env's from the 1 month and 3 month isolates were primarily cross-reactive (62%) with a small percentage being specific only for env from the 1 month isolate (19%) or the 3 month isolate (19%) (figure 14). However, at 7 months of age, although the percentage of 1 month-env specific pCTL remained the same (16%), the cross-reactive pool decreased by nearly half (35%) and the 3 month-env specific pCTL percentage increased to more than double (48%). Curiously, at 1 year of age, when the patient recognized heterologous env, the majority of the pCTL were cross-reactive either to all three env's (19%) or to combinations of two. However, the percentage of pCTL specific only for the 1 month env remained the same throughout (17%).

Figure 11. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL in cord blood (A) and at 6 months (B) of patient VI-06.

A.




B.

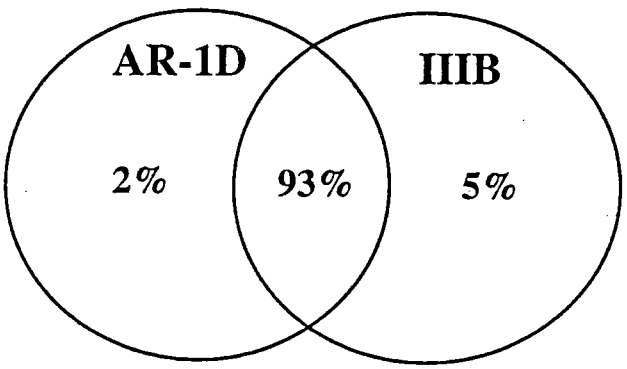
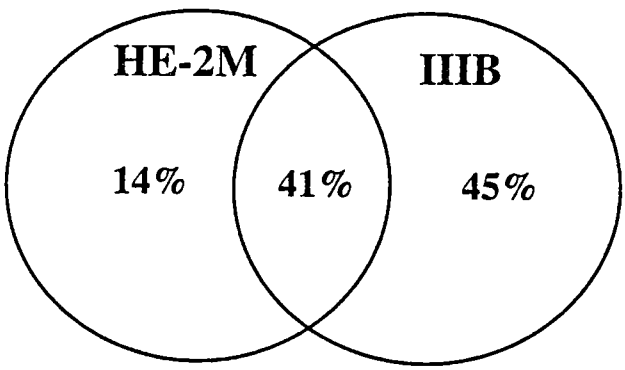


Figure 11. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL in cord blood (A) and at 6 months (B) of patient VI-06.

Individual wells set up in limiting dilution from PBMC from patient VI-06 from cord blood and 6 months of age were analyzed separately for cross-reactivity to both env-expressing targets used in the assay. Precursor frequencies of cross-reactive CTL were obtained as described in the Materials and Methods section and the percentages of cross-reactive and singly-reactive pCTL were calculated based upon these frequencies.

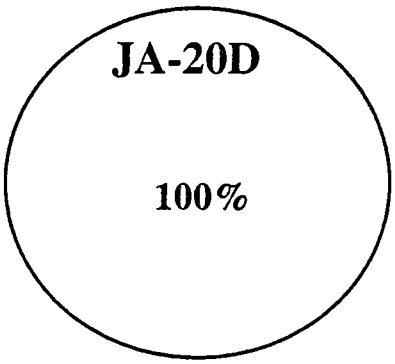
Figure 12. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL at 12 months of patient VI-05.




Individual wells set up in limiting dilution from PBMC from patient VI-05 from 12 months of age were analyzed separately for cross-reactivity to env-expressing targets used in the assay. Precursor frequencies of cross-reactive CTL were obtained as described in the Materials and Methods section and the percentages of cross-reactive and singly-reactive pCTL were calculated based upon these frequencies.

Figure 13. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL at 6 months (A) and 19 months (B) of age of patient VI-08.

A.




B.

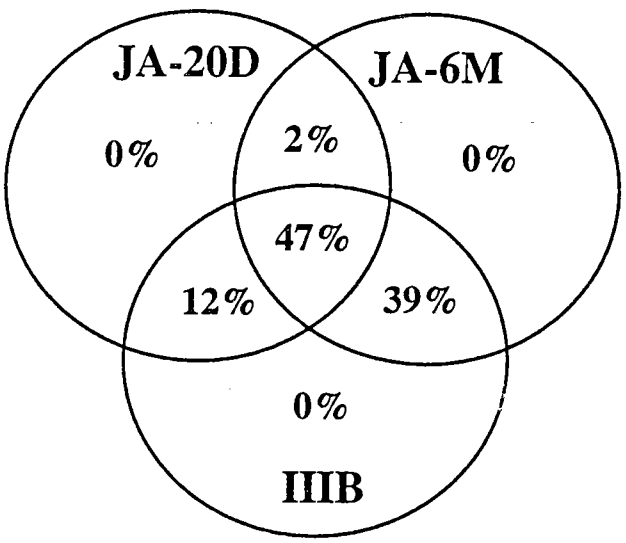
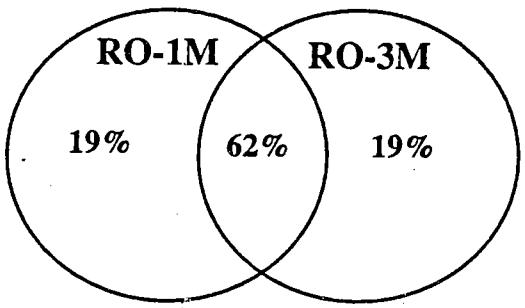


Figure 13. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL at 6 months (A) and 19 months (B) of age of patient VI-08.

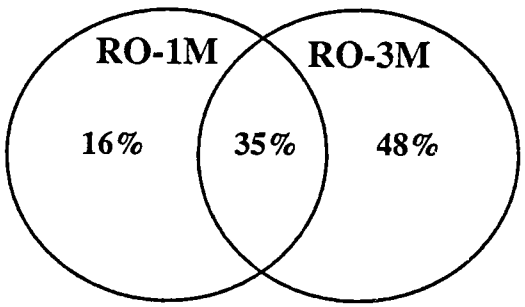
Individual wells set up in limiting dilution from PBMC from patient VI-08 from 6 and 12 months of age were analyzed separately for cross-reactivity to env-expressing targets used in the assay. Precursor frequencies of cross-reactive CTL were obtained as described in the Materials and Methods section and the percentages of cross-reactive and singly-reactive pCTL were calculated based upon these frequencies.

Figure 14. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL at 3 months (A), 7 months (B) and 12 months (C) of age from patient VI-11.

A.




B.




C.

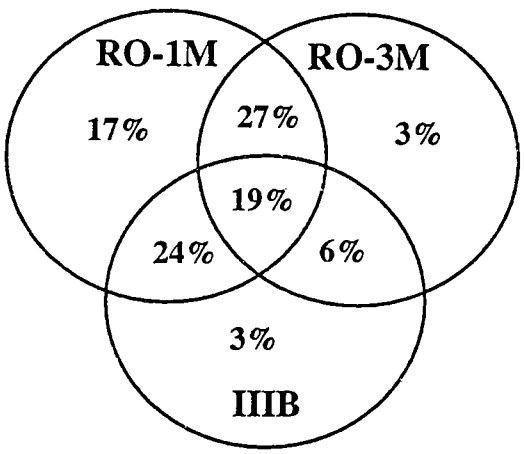


Figure 14. Percent autologous env-specific, IIIB env-specific and cross-reactive pCTL at 3 months (A), 7 months (B) and 12 months (C) of age from patient VI-11.

Individual wells set up in limiting dilution from PBMC from patient VI-11 from 3, 7 and 12 months of age were analyzed separately for cross-reactivity to env-expressing targets used in the assay. Precursor frequencies of cross-reactive CTL were obtained as described in the Materials and Methods section and the percentages of cross-reactive and singly-reactive pCTL were calculated based upon these frequencies.

## E. Precursor Frequency Analysis of CTL and against HLA-A2 restricted epitopes within PBMC of patients VI-06 and VI-08.

Patients VI-06 and VI-08 both possess the HLA-A2 haplotype to which several env CTL epitopes have been reported to be restricted (8, 87). These epitopes were primarily based upon IIIB env sequences. Taking advantage of this, the ability of patient VI-06, who had a very early group-specific CTL response, to recognize three of these published epitopes was examined. Sequence analysis of this patient's env clones in regions containing these epitopes was also performed. The same was done for patient VI-08. Peptides containing these epitopes were used to pulse targets, and pCTL were measured by LDA. Table 11 shows the sequences and locations of the epitopes and the peptides used to measure pCTL in this assay. PBMC were examined for pCTL from patient VI-06 at 3 months of age against these three peptides. Two of the peptides, 1923 and V3-1, were recognized at a range of 91-357 pCTL/$10^6$ PBMC (figure 15 and table 12). The CTL precursor frequency against peptide 1930 was not significantly higher than background. Sequence analysis of these epitopes within env clones derived from this patient at 1 day of age reveals complete conservation of sequence with the published epitopes within epitopes 1923 and 1930 (figure 16) and a relatively high degree of conservation within that of V3-1. However, sequence analysis of env clones generated from this patient at 6 months of age show a greater amount of divergence from the 1 day clones within the epitope V3-1 (figure 17). In contrast, these clones show very little divergence within epitopes 1923 and 1930 from the 1 day clones. Although patient VI-08 displayed IIIB responsiveness by at least 19 months of age, PBMC taken from this patient at 15 months of age did not demonstrate any measurable pCTL to any of these A2-restricted epitopes, even though there was almost complete conservation between the epitope sequences published and that of the patient's env clones derived from 20 days (figure 18) and 6 months of age (figure 19). Sequence

variation was only observed within the V3-1 epitope within some of the 6 month env clones to a very minor extent.

Table 11. A-2 restricted epitopes

| *Epitope** | *Amino Acids ‡* | *Peptide§* | *Epitope Name* |
|---|---|---|---|
| LWVTVYYGVPVWKEATTTLFCA | 34-55 | VPVWKEATTTLFCASDAKAY | (1923) |
| WDQSLKPCVKLTP | 112-124 | WDQSLKPCVKLTPLCVTLNC | (1930) |
| SVEINCTRPNNNTRKSI | 291-307 | SVEINCTRPNNNTRKSI | (V3-1) |

Epitopes 1923, 1930 and V3-1 are known CTL epitopes.

* The amino acid sequences of the epitopes reported by Clerici et al (87) and Dadaglio et al (8).

‡ Amino acid location of the epitope based upon the IIIB strain of HIV-1

§ Amino acid sequences of the peptides used in our study to measure pCTL frequencies directed at these epitopes

Table 12. Precursor frequency of CTL recognizing A2-restricted peptides of patient VI-06 at three months of age.

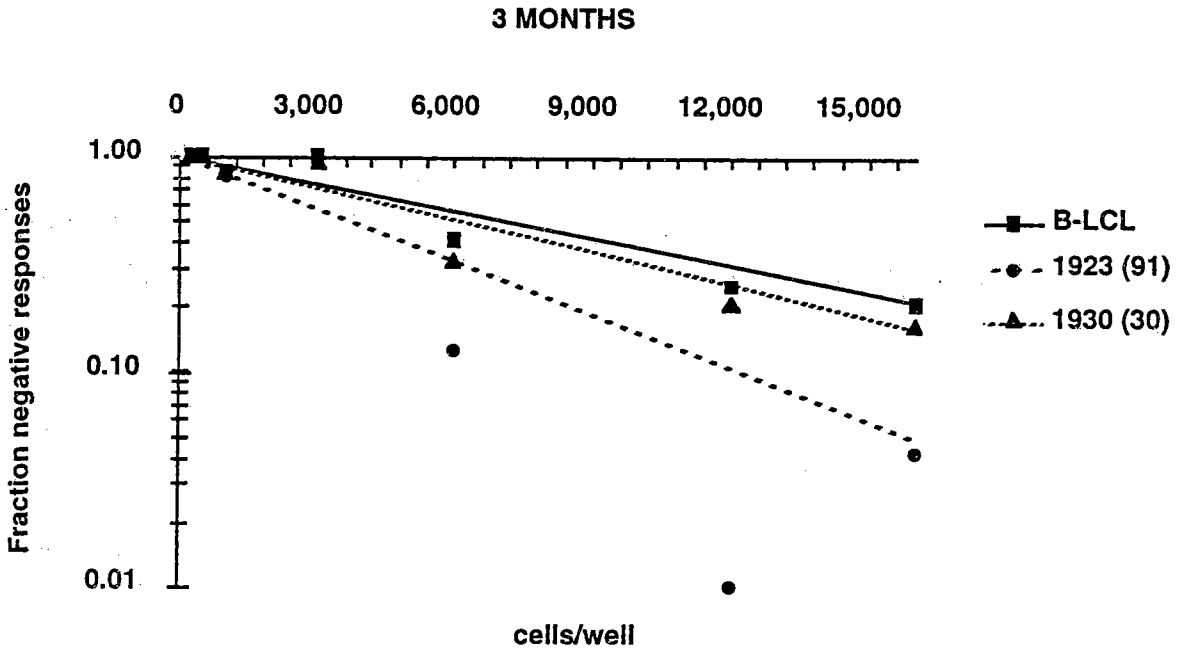
| *Peptide* | *pCTL/10$^6$ PBMC* |
|---|---|
| B-LCL* | 98 (75-129) |
| 1923 | 189 (146-244) |
| 1930 | 128 (98-167) |
| B-LCL* | 26 (17-40) |
| V3-1 | 383 (290-505) |

Precursor frequencies of CTL were measured by LDA in PBMC of patient VI-06 at 3 months of age against the peptides described in table 13. Target cells were pulsed with peptides as described in the Materials and Methods section. A separate assay was performed to determine the precursor frequency of CTL recognizing the V3-1 epitope. The frequencies of pCTL recognizing autologous B-LCL in the absence of peptide were quantitated to determine background pCTL frequencies.

* B-LCL values are from two separate assays

Figure 15. Precursor frequency analysis against 1923 and 1930 (A) and V3-1 (B) of patient VI-06 at 6 months of age

A.




B.

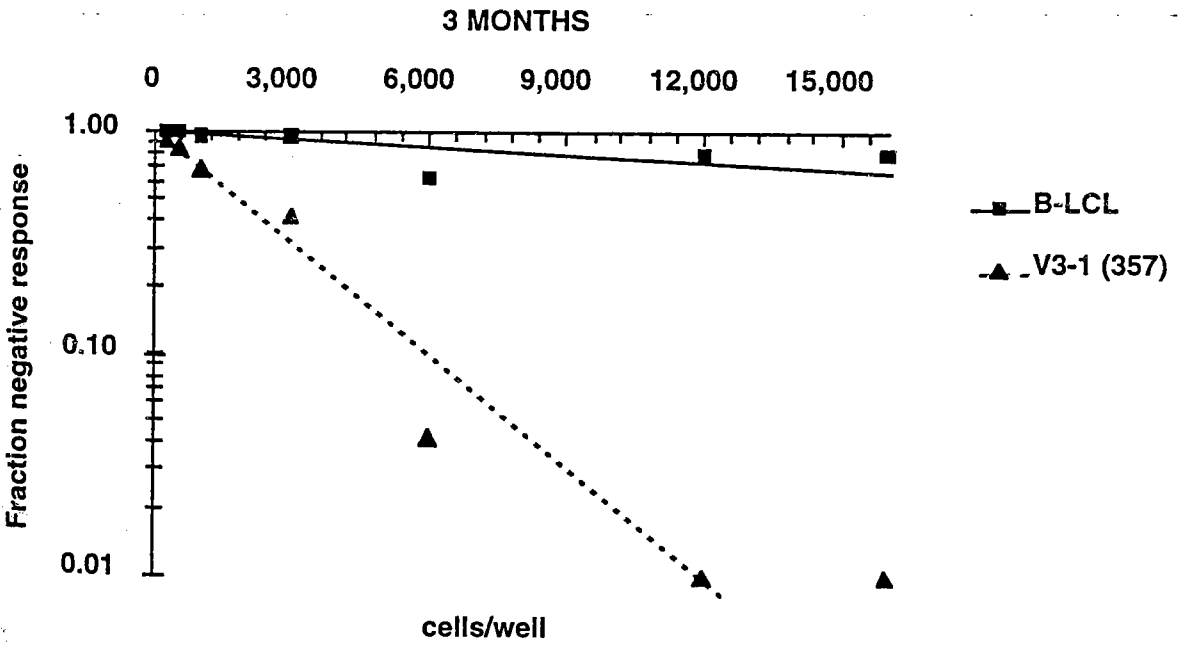


Figure 15. Precursor frequency analysis against 1923 and 1930 (A) and V3-1 (B) of patient VI-06 at 6 months of age

CTL precursors were quantitated using limiting dilution assays followed by in vitro stimulation of PBMC from 3 months of age. HIV-1 specific frequencies were calculated as described in the Materials and Methods section. CTL precursor frequencies were measured against autologous B-LCL in the absence of peptide and B-LCL pulsed with peptides corresponding to epitopes 1923, 1930 and V3-1. Numbers in parantheses are pCTL values with background subtracted. Spontaneous release was < 30% for all targets shown. The cut-off for a positive response was 10% for the two assays.

Figure 16. Sequence analysis of A2-restricted CTL epitopes within env clones derived from patient VI-06 at 1 day of age.

```
Published epitope sequence:   LWVTVYYGVPVWKEATTTLFCA
                  pAR-1-11    ......................
                  pAR-1-12    ......................
                  pAR-1-13    ......................
                  pAR-1-15    ......................
                  pAR-1-20    ..........
Published epitope sequence:   WDQSLKPCVKLTP
                  pAR-1-11    .............
                  pAR-1-12    .............
                  pAR-1-13    .............
                  pAR-1-15    .............
                  pAR-1-20    .............
Published epitope sequence:   SVEINCTRPNNNTRKSI
                  pAR-1-12    ..T...........G.
                  pAR-1-13    P.T...........G.
                  pAR-1-15    ..T...........G.
```

Nucleotide sequences were obtained from env genes ligated into the pCR3 (InVitrogen) backbone. Plasmid DNA was extracted from transformed bacteria and purified using acid-phenol as described in the Materials and Methods section. Double-stranded DNA sequencing was performed using a modification of the Sequenase Version 2.0 (United States Biochemical) protocol described in the Materials and Methods section. Both strands of DNA were sequenced using upstream and downstream primers and DNA sequences were read by hand.

Figure 17. Sequence analysis of A2-restricted CTL epitopes within env derived from patient VI-06 at 6 months of age.

```
Published epitope sequence:   LWVTVYYGVPVWKEATTTLFCA
                  pAR-2-2     ......................
                  pAR-2-3     ......................
                  pAR-2-7     ......................
                  pAR-2-8     ...S..................
                  pAR-2-9     ......H...............
                  pAR-2-10    ......................
Published epitope sequence:   WDQSLKPCVKLTP
                  pAR-2-2     .............
                  pAR-2-3     .............
                  pAR-2-7     .............
                  pAR-2-8     .............
                  pAR-2-9     .............
                  pAR-2-10    .............
Published epitope sequence:   SVEINCTRPNNNTRKSI
                  pAR-2-1     T.N.S....G....R..
                  pAR-2-2     T.N.S....G....R..
                  pAR-2-3     T.N.S....G....R..
                  pAR-2-4     T.N.S....G....R..
                  pAR-2-7     T.N.S....G....RRL
                  pAR-2-8     T.N.S....G....R..
                  pAR-2-9     T.N.S....G....R..
                  pAR-2-10    T.N.S....G....R..
```

Figure 18. Sequence analysis of A2-restricted CTL epitopes within env derived from patient VI-08 at 20 days of age.

```
Published epitope sequence:   LWVTVYYGVPVWKEATTTLFCA
                  pJA-5-2     ......................
                  pJA-5-3     ......................
                  pJA-5-4     ......................
Published epitope sequence:   WDQSLKPCVKLTP
                  pJA-5-2     .............
                  pJA-5-3     .............
                  pJA-5-4     .............
Published epitope sequence:   SVEINCTRPNNNTRKSI
                  pJA-5-2     .................
                  pJA-5-3     .................
                  pJA-5-4     .................
                  pJA-5-9     .................
```

Figure 19. Sequence analysis of A-2 restricted CTL epitopes within env derived from patient VI-08 at 6 months of age.

```
Published epitope sequence:   LWVTVYYGVPVWKEATTTLFCA
                  pJA-6-2     ..............T.....R.
                  pJA-6-3     ......................
                  pJA-6-4     ......................
                  pJA-6-8     ......................
Published epitope sequence:   WDQSLKPCVKLTP
                  pJA-6-2     .............
                  pJA-6-4     .............
                  pJA-6-8     .............
Published epitope sequence:   SVEINCTRPNNNTRKSI
                  pJA-6-2     .............S.KY
                  pJA-6-4     .................
                  pJA-6-7     .................
                  pJA-6-9     ...........S.....
                  pJA-6-10    ..............VY
```

F. ADCC antibody titers against autologous and heterologous env proteins within patient plasma.

Previous work in our laboratory has demonstrated the lack of measurable titers of ADCC antibodies within the first year of life in the majority of vertically infected infants. Again, the env gene used for these studies was IIIB. Therefore, the env recombinant vaccinia vectors derived from clinical isolates were used to determine whether there were measurable titers present to autologous env sequences during early infancy. Plasma from patients VI-06, VI-05, VI-08 and VI-11 was titrated against IIIB and autologous env at four different time points, which ranged over the ages of 2 months to 24 months. The antibody titers present from 2 months to approximately 6 months of age were indistinguishable from maternal antibody. Therefore, antibody measured subsequent to 6 months was considered to be infant-derived.

Not surprisingly, plasma from patient VI-06 was strongly group-specific, demonstrating high titers to both autologous env and IIIB (figure 20). Titers rose approximately 100-fold from 2 months of age to 6 months of age, against autologous and heterologous env, indicating vigorous antibody production during these early months by the patient. These high titers were maintained through at least 25 months of age.

ADCC antibody titers of patient VI-05 were type-specific from 6 to 20 months of age, with responses only to autologous env derived from 2 months of age (figure 21). Maternal ADCC antibody titers were measured against both IIIB and the patient's 2 month autologous isolate at 2 months of age at equivalent titers. Interestingly, the autologous env derived from virus present at 6 months of age was not recognized by ADCC mediating antibodies (nor was it recognized by maternal antibody). ADCC antibodies from 2 to 6 months of age were maintained at low titers but then dropped 10-fold lower between 6

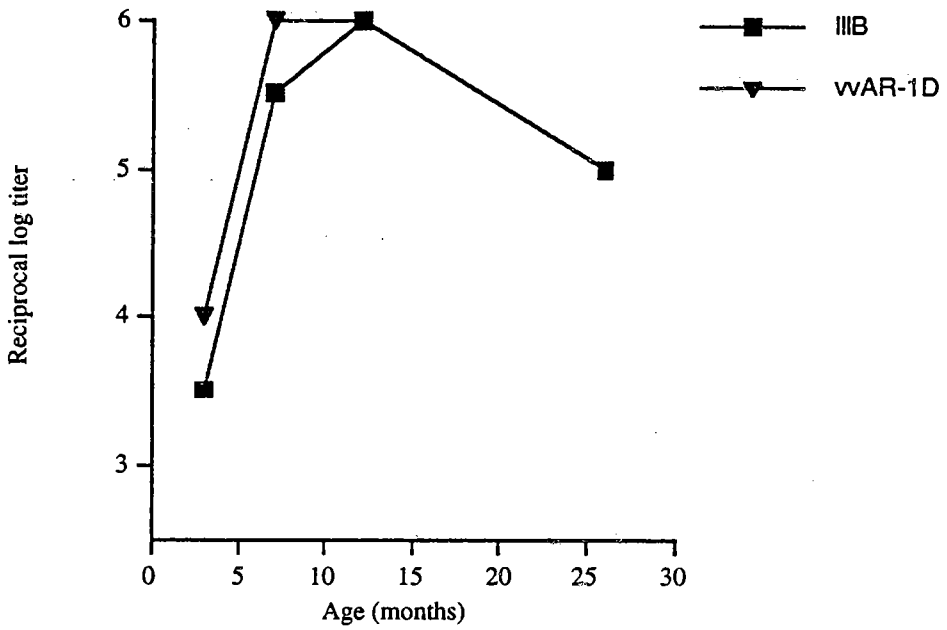
months and 12 months and then were maintained just above the level of detection for the assay from 12 to 20 months.

High ADCC titers could be measured in the plasma of patient VI-08 prior to 6 months of age against both IIIB env and, curiously, VI-08's autologous 6 month env but not the 20 day env (figure 22). Titers to IIIB were 10-fold lower than that against autologous env. These titers dropped precipitously by 6 months, at which time point only very low 20 day autologous env titers could be detected. From 6 months to 24 months of age, ADCC titers continued to rise, again, only against the 20 day autologous env.

Detectable ADCC titers were completely lacking in the plasma of patient VI-11 from 2 to 6 months of age (figure 23). However, between 6 and 12 months of age, ADCC titers to all three env's (autologous 1 month, 3 month and IIIB) could be measured. Titers were at least 100-fold greater to autologous env derived from 3 months of age at the 24 month time point.

Lack of ADCC titers against the recombinant env-expressing vectors could be explained by either an overall inability of a certain env to be a target for ADCC or by an intrinsic inability of the patient to generate ADCC antibody to that particular env. To test for the former, titers of ADCC antibody were measured in plasma that had been pooled from HIV-1 infected individuals with hemophilia against all of these patient-derived env's. High titers were detected in this pooled plasma against all of the clinically-derived env's (figure 24).

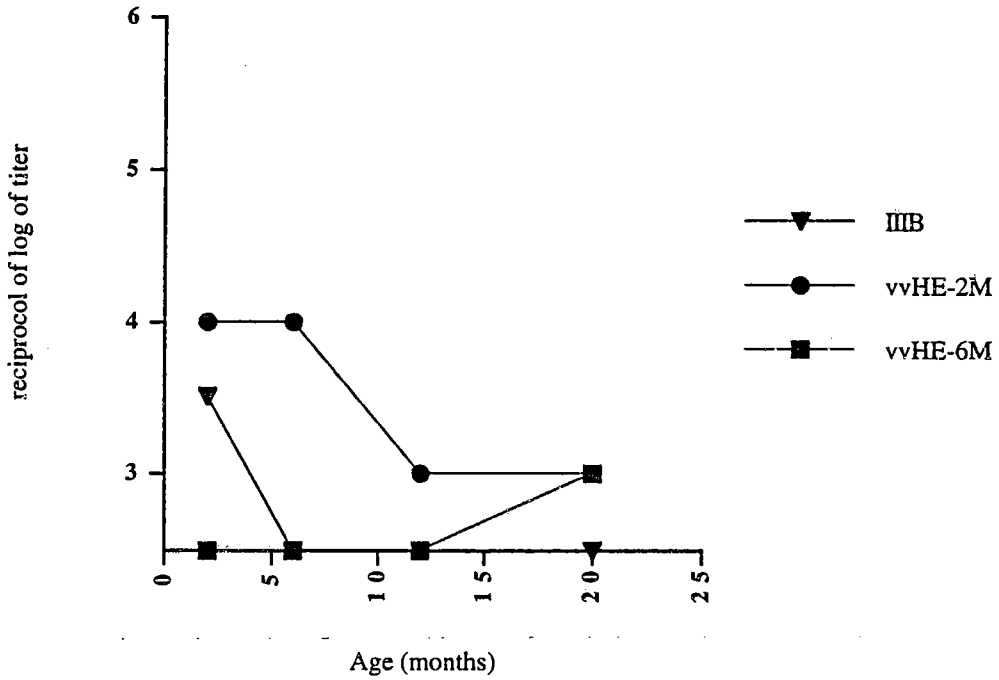
Figure 20. ADCC titers of patient VI-06 over time against autologous and IIIB env.




ADCC antibody titers were measured in plasma of patient VI-06 at 2, 6, 12 and 24 months of age as described in the Materials and Methods section. The limit of detection of the assay is a titer of $10^{-3}$, therefore, any data point below 3 on the Y-axis is interpreted as lack of ADCC antibody titer. The env-expressing targets are autologous B-LCL infected with env-recombinant vaccinia viruses expressing IIIB env (IIIB) and env derived from an HIV-1 isolate of patient VI-06 at 1 day of age (vvAR-1D).

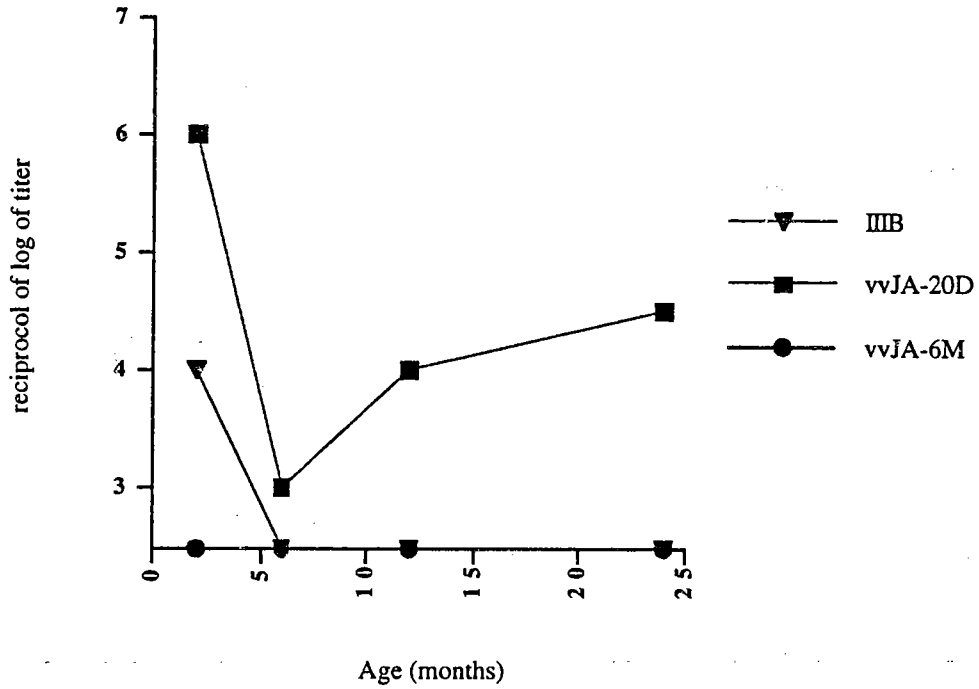
Titers below 3 represent values below detection of the assay.

Figure 21. ADCC titers of patient VI-05 over time against autologous and IIIB env.



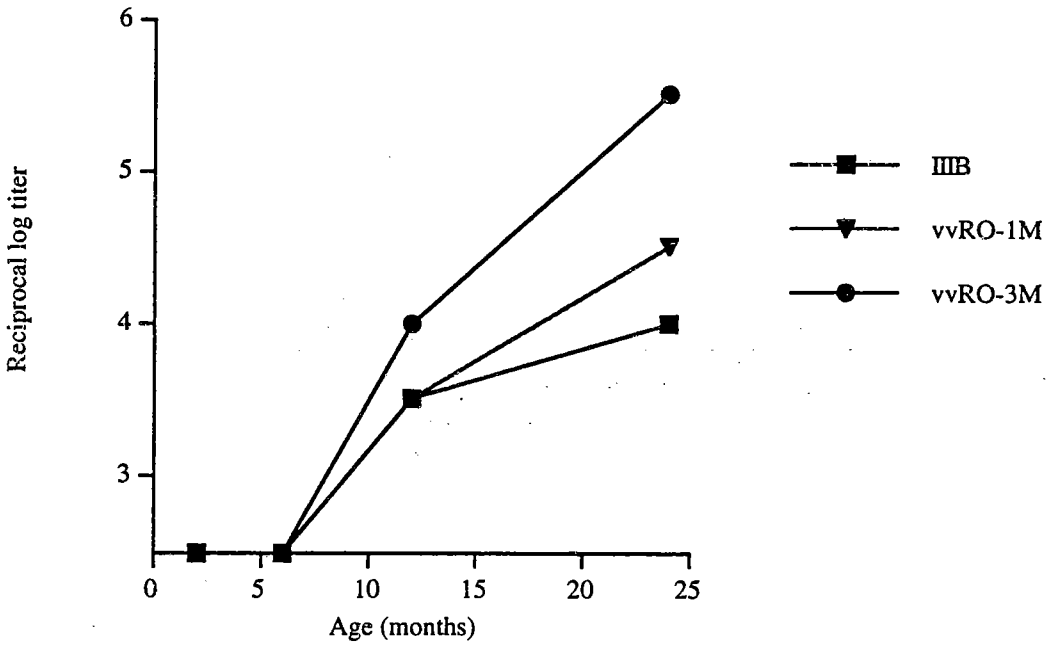

ADCC antibody titers were measured in plasma of patient VI-05 at 2, 6, 12 and 20 months of age as described in the Materials and Methods section. The limit of detection of the assay is a titer of $10^{-3}$, therefore, any data point below 3 on the Y-axis is interpreted as lack of ADCC antibody titer. The env-expressing targets are autologous B-LCL infected with env-recombinant vaccinia viruses expressing IIIB env (IIIB) and envs derived from an HIV-1 isolate of patient VI-05 at 2 months (vvHE-2M) and 6 months (vvHE-6M) of age.

Figure 22. ADCC titers of paitent VI-08 over time against autologous and IIIB env.



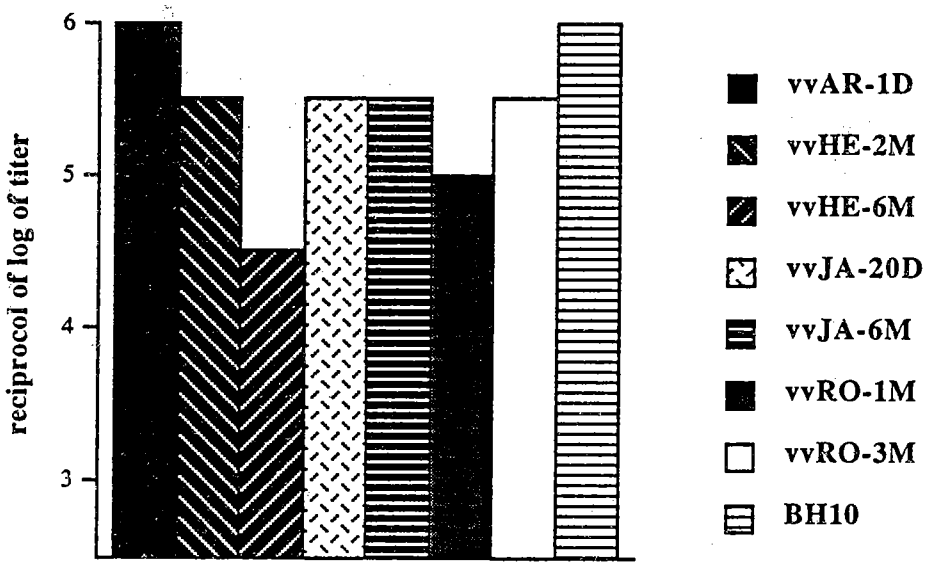

ADCC antibody titers were measured in plasma of patient VI-08 at 2, 6, 12 and 24 months of age as described in the Materials and Methods section. The limit of detection of the assay is a titer of $10^{-3}$, therefore, any data point below 3 on the Y-axis is interpreted as lack of ADCC antibody titer. The env-expressing targets are autologous B-LCL infected with env-recombinant vaccinia viruses expressing IIIB env (IIIB) and envs derived from an HIV-1 isolate of patient VI-08 at 20 days (vvJA-20D) and 6 months (vvJA-6M) of age.

Figure 23. ADCC titers of patient VI-11 over time against autologous and IIIB env.




ADCC antibody titers were measured in plasma of patient VI-11 at 2, 6, 12 and 24 months of age as described in the Materials and Methods section. The limit of detection of the assay is a titer of $10^{-3}$, therefore, any data point below 3 on the Y-axis is interpreted as lack of ADCC antibody titer. The env-expressing targets are autologous B-LCL infected with env-recombinant vaccinia viruses expressing IIIB env (IIIB) and envs derived from an HIV-1 isolate of patient VI-11 at 1 month (vvRO-1M) and 3 months (vvRO-3M) of age.

Figure 24. ADCC titers of pooled positive plasma against patient-derived env's and IIIB




ADCC antibody titers were measured in plasma which was pooled from HIV-1 infected individuals with hemophilia and was previously demonstrated in our laboratory to have broad recognition of HIV-1 envs of different strains. Targets were B-LCL infected with IIIB-env-expressing vaccinia virus and B-LCL infected with recombinant vaccinia viruses expressing each of the env genes derived from the HIV-1 isolates of patients VI-06, VI-05, VI-08 and VI-11.

# CHAPTER IV: DISCUSSION

## A. Env clones derived from low passage virus co-culture are relevant to the immune response.

In order to minimize the chance of amplifying defective HIV-1 env sequences, we chose to construct the env-expressing recombinant vaccinia vectors with env genes amplified from cultured virus. Many studies have demonstrated the outgrowth of predominant variants of limited diversity in viral co-culture which were not predominant in vivo at the time point at which the culture was established (17, 89-91). These variants selected for in culture may be minor variants in vivo for a number of reasons: they may be suppressed by an active immune system, anti-retroviral therapy or they are a newly evolved quasispecies which will emerge as a predominant variant at a later time point. Evidence by Delwart et al (17) has demonstrated the latter possibility in heteroduplex tracking analyses in which cultured virus was observed to be a minor variant in vivo at the time of culture but at a later time point was present in abundance. Delwart's study suggests that viral variants derived from culture are also present in vivo at levels necessary to stimulate immunity. The absence of anti-retroviral therapy in these patients at each of the time points from which the env vaccinia recombinants were generated supports the notion that these variants were not suppressed in vivo and expanded in vitro due to absence of the drug. Finally, 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. Additionally, studies which have compared the selection of variants in culture of limited diversity to that present in vivo have maintained these viral cultures out to at least 28 days

(90). The env genes used to generated recombinant vv in our studies were amplified from virus which had been cultured for less than a week and passaged either once or twice which may have minimized the in vitro selection of variants.

Alternate sources of template for amplification of viral env sequences include proviral DNA present in uncultured PBMC or viral RNA present in plasma virions. Variants present within uncultured PBMC are considered to be a collection of both replicating virus and "graveyard" sequences of defective viruses. Thus, generating env clones from circulating, uncultured PBMC was less desirable due to the risk of cloning these defective sequences. The use of viral RNA as template was also less desirable but from the standpoint of practicality. Amplification of the entire env gene would have had to include a reverse transcription step followed by DNA PCR. Generating a PCR product from reverse transcription of a template of this size in sufficient quantities to clone would have been technically infeasible. Therefore, in the interest of obtaining biologically relevant env clones which would be used to gauge immunity in patients VI-06, -05, -08 and -11, we chose viral co-culture as a source of these clones. Furthermore, studies by Wolinsky et al support the notion that concern over variant selection of early infant viral isolates in culture may be unwarranted. Sequence comparison of the V3 and V4-V5 regions of env within 2 to 4 month old infants by this group revealed little diversity of viral variants derived from PBMC (18). Given this level of homogeneity in PBMC-derived virus at these early time points, it is reasonable to assume that cultured variants from these same time points would be representative of the in vivo variant population.

## B. Diversity of patient proviral DNA sequences from HIV-1 IIIB.

Group-specific CTL responses can be detected as early as pre-seroconversion in adults using heterologous viral gene products (29, 36, 92) as targets. Yet we detect only

type-specific CTL responses in infants during the first year of life. With the multitude of HIV-1 CTL epitopes that have been mapped to env (93-95) it is difficult to imagine that enough overall heterogeneity exists between heterologous and autologous env gene products within this infant population to abrogate recognition of all of these regions. Indeed, the degree of diveristy between the infant-derived env sequences and IIIB (BH10), as determined by heteroduplex analysis, was minor (in the range of 4-5%). Nor could a shift in the env population toward greater homology with IIIB over time be an explanation for the appearance of IIIB-specific CTL by 1 year of age in these patients. For that matter, patients VI-08 and VI-11 demonstrated minor heteroduplex mobility changes within their later isolates that would suggest further divergence from IIIB.

Although many CTL epitopes have been described within env, their relevance to CTL recognition in vivo is questionable. Many of these epitopes are detected in assays in which microgram amounts of peptide are used in the peptide-pulsing of targets (8, 10, 87). High densities of these peptides on the target cells may result in target cell lysis by low affinity/specificity CTL which may not interact with in vivo targets strongly enough to effectively contribute to the CTL response. CTL clones obtained by limiting dilution have also been used extensively to map HIV-1 epitopes (33, 96-98). Although these studies provide information concerning class I binding motifs within HIV-1 proteins, the actual representation of these CTL clones in vivo remains unclear. Therefore, in spite of the presence of many potential CTL epitopes, a small number of CTL epitopes may dominate the immune response at a given point of infection. If this were true, the overall sequence diversity between two env genes as examined by heteroduplex formation analysis may be relatively uninformative in predicting whether the CTL response to their gene products would be group or type-specific. Detecting differences within known HLA-restricted CTL epitopes by DNA sequence analysis would better indicate the broadness of the immune

response. However, if immunologically dominant regions are present, this would need to be done in retrospect after mapping the dominant epitope.

C. HIV specific pCTL were detected in 3 of 4 infants in early infancy

Luzuriaga et al (64, 68, 99), using IIIB-based gene products as targets, have reported the lack of circulating HIV-1 specific CTL from unstimulated PBMC and lack of detectable CTL precursors from nonantigen-specific stimulation of PBMC within the first year of life in vertically infected infants. These CTL responses subsequently appear, following approximately one year of age. The presence of HIV-1 specific CTL in vertically-infected patients older than 12 months has been well-documented by several investigators (65-67). Each of these studies used laboratory-based viral gene products to measure CTL responses. The lack of HIV-$1_{IIIB}$ env-specific CTL precursors within the PBMC of patients VI-05, VI-08 and VI-11 prior to 12 months of age is in agreement with these results. In contrast, a study done by Cheynier, et al (100) detected HIV-$1_{IIIB}$ specific CTL in three out of six infants less than 12 months of age within circulating, unstimulated PBMC. However, the target cells used in these assays were P815 cells co-transfected with HLA-A2 and either HIV-1 env, gag or nef. High expression of these transfected genes in addition to a lengthy incubation time of effectors and targets (18 hours) could allow for a less specific assay in which an "antigen-loaded" target could be lysed by CTL of weak affinity. Thus, the results obtained in this study should be interpreted with caution as they may overestimate the lytic potential of these CTL in vivo.

The results of this study demonstrate the necessity of using autologous virus for the detection of HIV-1-specific CTL responses in early infancy in infants infected with HIV-1. Prior lack of detection of IIIB-env-specific pCTL in these infants was misleading in suggesting an inability of young infants to mount HIV-1-specific cellular immune

responses. It is now evident that in the absence of detectable CTL responses to laboratory HIV-1 strains, young infants possess CTL which can recognize autologous viral gene products and are present at frequencies at which laboratory-strain specific CTL have been measured in older vertically infected children (67). In 3 infants, the detection of pCTL recognizing target cells expressing early isolate env preceded the detection of pCTL recognizing target cells expressing IIIB env. These type-specific pCTL detected in early infancy were replaced by cross-reactive, group-specific CTL by 6 to 12 months of age. In a fourth infant, pCTL recognizing target cells infected with HIV-1 IIIB env and early isolate env were simultaneously detected at 12 months of age.

It is important to note that many technical problems are inherent in the construction of the assays employed for pCTL quantitation. These technical difficulties must be kept in mind in the careful interpretation of the results. For example, only a limited quantity of PBMC can be obtained from a young infant. These PBMC, following cryopreservation, are also fairly fragile which further lowers the yield of PBMC after thawing. Although there were sufficient numbers of PBMC to perform the assays described above, we could not deplete for NK cells. As a result, background lysis (although corrected for in the mathematical analysis) was variably high between time points taken from the same patient. To control for this variability, the same donor was used as a source of feeder cells at each time point and the pCTL from each time point was measured on the same day with the same target B-LCL. Despite these precautions, increased background lysis could still be detected in PBMC from particular time points. This could be due to the original composition of cells of lytic potential within the starting PBMC population. Although using the same batch of B-LCL targets for each time point decreased the variability of susceptibility of target cells to lysis, it was technically difficult to mix target and effector cells from each time point at the same time. Therefore, there was a time differential between the application of target cells to effectors from one time point in relation to the other. The time lag between the two

applications could also contribute to higher background lysis due to increased target fragility over time. The results of this study are therefore useful in determining whether a CTL response is present within PBMC from a particular time point if pCTL quantities exceed background. However, we are limited in the comparison of absolute quantities of pCTL able to recognize a specific env gene product from two different time points due to the background lysis variability between these time points.

PBMC of patient VI-06 taken from cord blood appeared to have some reactivity to autologous env taken from a 1 day isolate. While this patient was also shown in a previous study to demonstrate measurable pCTL within PBMC of cord blood against HIV-$1_{IIIB}$ gag gene products (68), the autologous env pCTL frequency was not significantly higher than background lysis due to slightly overlapping 95% confidence intervals. However, we believe the increased pCTL values obtained against 1 day autologous env are real. Maternal lymphocytes within cord blood could mediate cytolysis, however HLA typing demonstrated only fetal lymphocytes within the cord blood. Even more surprising was the demonstration of a group-specific CTL response within the PBMC of this patient as early as 6 months of age. Vigorous CTL activity to both HIV-$1_{IIIB}$ env and gag gene products within PBMC measured directly in bulk without prior in vitro stimulation was seen during early infancy (68). Positive cord blood viral cultures and DNA PCR from this individual supports the diagnosis of in utero infection. If viral heterogeneity is considered to contribute to a broader, more-group specific CTL response, than one could theorize that this particular patient's immune system, by 6 months of age, had encountered an array of variants. In support of this, HIV-1 RNA copy number had risen from 49,000 copies/ml at birth to 1,300,000 copies/ml by 4 months of age. Even clones that were derived from this patient cultured virus from at 1 day of age demonstrated a greater heterogeneity than that seen in clones from this patient's virus isolate at 6 months of age or in clones of viruses from the other three patients at any of the time points studied. At 1 month of age, the

patient received anti-retroviral monotherapy and by 5 months of age was being treated with two anti-retrovirals concommitantly perhaps accounting for the decrease in viral heterogeneity seen in the 6 month clones. One possible explanation for the degree of heterogeneity seen so early in infancy could be infection which occurred early enough in utero to allow for the generation of many variants. In the majority of cases, primary infection involves the successful outgrowth of perhaps one to only a few viral variants (16-18, 101) due to the transmission of a single or a few viral variants. If the variants within VI-06 were derived from a single progenitor and became heterogeneous during gestation it would seem that selective pressure was applied during this time to the virus to diversify. Possible contributors to selection would be maternal antibody which can traverse the placental barrier or fetal immune responses. On the other hand, it is possible that in the case of this infant, early in utero infection was not so much a factor as, perhaps, an unusually large viral inoculum. In this case, one might speculate, that several variants might have the ability to be successfully transmitted. In fact, co-infection by more than one variant within acute adult seroconverters has been documented through heteroduplex mobility and DNA sequencing analysis (17, 102) Alternatively, the class I alleles of this patient in combination with the TCR repertoire may allow for presentation and recognition of a wider assortment of CTL epitopes than what is seen with most patients. Whatever the case may be, this infant who was capable of early group-specific immune responses also possessed an unusually high early viral burden. Although this patient's immune responses were vigorous, it suggests that viral replication outstripped the immune response very early on.

Patient VI-05 had measurable CTL responses directly from PBMC against HIV-$1_{IIIB}$ env by 11 months of age as determined previously in our laboratory. In contrast with patient VI-06, this patient did not demonstrate pCTL within cord blood to any of the envs tested. Although both patients were infected in utero, the differences in CTL responses

could relate to the factors described for patient VI-06, namely timing of infection or viral dose at transmission. Interestingly pCTL directed against this patient's 6 month env could not be detected at 12 months of age although there were significant frequencies of CTL against autologous 2 month and IIIB envs. Lack of CTL recognition could be due to viral escape from CTL recognition of the entire env gene. Alternatively, divergence of this patient's viral isolates by 12 months of age could have resulted in a CTL epitope sequence(s) which was less similar to that present at 6 months and more like IIIB. The CTL stimulated in response to the virus present at 12 months may not have specificity for this earlier env.

As with patient VI-05, PBMC of patient VI-08 had previously been examined for directly measurable CTL activity against HIV-$1_{IIIB}$ env and gag gene products in bulk assays and in LDA assays following nonspecific in vitro stimulation. Env and gag-specific CTL were not detected by either method until 11 months of age at which time very low env lysis could be measured directly. Detection of pCTL at 6 months of age against VI-08 autologous env taken from the 20 day isolate demonstrated the ability of this patient to mount a cellular HIV-1 specific immune response in early infancy. Additionally, it was observed that at 6 months of age, although this patient was able to recognize env from a 20 day viral isolate, pCTL to the env of the 6 month virus were undetected. A possible explanation for this result that at the time of sampling, the variant was not predominant in vivo but became so during the culturing process. Therefore, the amount of antigenic stimulation needed in vivo for CTL proliferation may not have reached threshold until a time point beyond 6 months. This idea is further supported by the fact that by 19 months, pCTL recognizing the 6 month env are readily detectable. Alternatively, the CTL epitope(s) within this env may represent escape mutants to which the pCTL at 6 months of age were unable to respond. By 19 months of age, a new env epitope may have become dominant which was present in all three envs.

In previous studies, patient VI-11 did not have directly measurable CTL responses in bulk within PBMC against IIIB env or gag gene products prior to the first year of life. Unlike patients VI-05 and VI-08 however, pCTL were detected by 3 months of age against IIIB gag by nonspecific in vitro stimulation in LDA but not against IIIB env. Recognition of both autologous env's derived from viral isolates at 1 month and 3 months of age by patient VI-11 at all three time points examined is not surprising when one considers how similar the two env genes appear by heteroduplex analysis. Heteroduplexes formed between pNL4-3 and these two env clones migrated at approximately the same speed although the lower band of the heteroduplex doublet formed between pRO-3M and pNL4-3 was slightly higher than the same band in the heteroduplex formed between pRO-1M and pNL4-3. This suggests a small degree of sequence heterogeneity between the two.

## D. Role of CTL in primary infection

Although HIV-1-specific pCTL are present during early infancy in vertically infected infants, their anti-viral role is unclear. Patient VI-06 demonstrated pCTL specific for both gag and possibly autologous env in PBMC of cord blood as well as IIIB env pCTL present within 3 weeks of life (68). CTL precursor frequencies against IIIB gag and env gene products were detected consistently over a period from birth to 16 months of age (68). Recent studies (29, 36) have correlated the presence of HIV-1-specific pCTL in adults during seroconversion with control of acute viremia. Yet, this patient's viral load was high at birth and continued to climb over the patient's first 4 months of age where it peaked at 1,300,000 RNA copies/ml of plasma. The ability to measure HIV-1-specific CTL responses in the PBMC of infected adults without prior in vitro stimulation is a hallmark of HIV-1 infection in this population. Although CTL precursors were readily detected in this patient during the first few months of age, CTL responses within

unstimulated PBMC were not examined until 4 months of age. By 4.5 months of age, this patient demonstrated measurable CTL responses against both env and gag gene products in PBMC which were not stimulated in vitro (68). Although the bulk CTL responses within this patient prior to 4 months of age is unknown, it is possible that the HIV-1-specific CTL which we have detected following in vitro stimulation may not be expanded to a sufficient extent in vivo for their direct detection. In fact, in vitro stimulation has been demonstrated to be necessary in order to detect HIV-1-specific CTL in vertically infected children older than 12 months of age (67). Limited expansion of HIV-1-specific CTL may relate to the naive state of the immune system in infants and may be prove less effective in controlling early viremia. Type-specific CTL responses could not be measured directly from unstimulated PBMC in these patients due to the limited number of cryopreserved cells available for study but would have been informative as to the possible role of CTL in HIV-1 infection of infants.

### E. Phenotype of effectors mediating target cell lysis in PFA

The phenotype of the effector cells in adults and children mediating cytolysis against env-expressing target cells in CTL assays has been reported to be a combination of CD8, T lymphocytes and CD16 (NK) lymphocytes which are presumably armed with ADCC mediating antibody via their Fc receptors (64, 67, 100, 103, 104). Determination of effector cell phenotype was accomplished primarily through depletion of cell populations using specific antibody and complement. However, this method can result in overall cell loss in addition to depletion of a particular cell phenotype. Therefore, due to the limited number of cells available for this study, NK cell depletion of patient PBMC prior to analysis was impractical. However, NK mediated cytolysis of env-expressing targets by ADCC can be ruled out with a great degree of certainty based upon ADCC antibody titers

specific for these same env targets at these same time points. For example, PBMC of patient VI-05 at 12 months of age had detectable pCTL to IIIB env in the absence of measurable ADCC antibody to this same env at this same time point. This lysis seen against IIIB env, therefore, most likely cannot be attributed to ADCC. The same is true for recognition of patient VI-08's pCTL of IIIB env and autologous 6 month env at 19 months of age and patient VI-11's pCTL recognition of 1 and 3 month autologous envs at 3 and 7 months of age. ADCC antibody titer was not detected against IIIB or autologous 6 month envs in patient VI-08's plasma at 19 months of age or autologous 1 and 3 month envs at 3 and 7 months of age in patient VI-11's plasma. In further support of the notion that NK lysis did not play a major role in cytolysis of targets in these assays, Buseyne et al demonstrated that cytolysis mediated by a PBMC effector population stimulated in vitro by similar means was uneffected by CD16 depletion (65). It is possible that there are NK cells armed with ADCC mediating antibody within these patient PBMC even though this antibody cannot be detected in plasma. However, considering that these titers are below detection, these would be expected to contribute to cytolysis to a rather minor degree. It is also possible that cytolysis could be due to direct NK cell lysis of env-expressing targets, however, it is unlikely that different envs would be differentially recognized by these cells. In addition, it is notable that env cytolysis did not occur in unstimulated bulk assays performed previously in our laboratory on these patients, suggesting that NK effector participation was minimal.

### F. Fluctuating patterns of recognition of autologous env sequences

The term "immunodominance" in the CTL response against HIV-1 has been used to describe two different situations: in one, the term applies to an antigenic property of the virus alone, in the other it applies to the interactive dynamics of the immune response with

viral replication. In the first, a region can be considered immunodominant within a viral protein if several CTL epitopes are present within it which are restricted by a variety of HLA class I haplotypes. CTL from most individuals with varying HLA class I alleles can recognize peptides from this immunodominant region. However, studies mapping immunodominant regions do not address whether the CTL response against this region represents a dominant response within a particular individual. Again, as described above, low affinity CTL could possibly contribute to this CTL response measured in vitro. An example of an immunodominant region within nef was described by Couillin et al (amino acids 73-144) which was recognized by CTL of most seropositive adults tested (10). However, they observed a population of patients whose CTL recognition of nef did not map to this region although they possessed the appropriate class I alleles for restriction. Sequence analysis of isolates from these patients revealed mutations within this region which reduced or eliminated CTL responsiveness. From this, one could speculate that CTL directed to peptides from this region may have comprised the dominant immune response to nef at one time and that immune pressure resulted in the mutations allowing for CTL escape. However, without a sequential study over time of CTL recognition of this peptide coupled with epitope sequencing of isolates, it is difficult to interpret this data with regard to a dominant immune response.

The second situation to which the term immunodominance is applied is that in which one CTL epitope within a viral gene product dominates the immune response to that viral protein (even in the presence of several potential CTL epitopes within the viral protein). Immunodominant peptides within viral gene products from various viruses have been described extensively in the murine system (105-109). Although demonstration of immunodominance within this model is facilitated by the inbred backgrounds of these animals coupled with a less diverse HLA class I repertoire than that seen in humans, information derived from murine models can be used to further our understanding of

human immunity. HIV-1 specific CTL responses directed against reverse transcriptase (110) and env (111-113) in mice have also been mapped to immunodominant epitopes. For example, Takahashi et al described an immunodominant epitope within the V3 loop of gp160 recognized by H-$2^d$ mice immunized by a recombinant vaccinia vector expressing the env gene of HIV-1 IIIB (111). H-$2^k$ mice similarly immunized were completely lacking in gp160 CTL responsiveness. The ability of the H-$2^k$ mice to make class II helper responses to gp160 (114) as well as vaccinia suggested that CTL unresponsiveness in this strain of mice was associated with a lack of env specific class I MHC molecule-restricted T-cell responses. CTL responsiveness in mice of a particular class I haplotype toward one predominant epitope could be mirrored in the human HIV-1 cellular immune response. For instance, Lieberman et al, using nonspecifically stimulated T cell lines from HIV positive individuals, mapped the CTL response against env and reverse transcriptase to one or two isolate-invariant epitopes (34).

Of course, with increasing variant complexity in HIV-1 infection, increasing numbers of CTL epitopes would probably be recognized with a dependence upon fluctuations in the variant population. Nowak et al has proposed a mathematical model which attepts to reflect the dynamics of CTL activity in response to these fluctuations in HIV-1 quasispecies (115). His modeling is based upon observations of HIV-1 gag-specific CTL activity followed for 55 months within two HIV-1 infected individuals with hemophilia. The peptide-specificity of the gag CTL response within one patient did not change over the course of the study. Additionally, almost all the viral variants of this patient sequenced over the course of this study retained this index sequence. In contrast, the second patient's gag-specific CTL response alternated at particular time points over the course of the study among three gag epitopes. Sequence analysis of this patient's variants over time within these three epitopes revealed one amino acid substitution within each. Gag specific CTL of this patient could also respond to these mutant epitopes. An

oscillation of the viral variants present over time was observed in this patient which seemed to correlate with a fluctuation of CTL responses against either wild type or mutant peptide specificities. At certain time points, only wild type or mutant peptide were recognized exclusively even though the variant carrying that sequence was present. Interestingly, the first patient remained well, whereas the second progressed to AIDS within 8 years. The model proposed by this study attempts to describe these oscillations within CTL responses and variant epitope sequences in mathematical terms. Within this model there is a prediction of competition between different epitopes which can result in immunodominance of one despite the presence of the other. This could happen in a situation in which there is a stronger CTL response to the first epitope compared to the second. Therefore, the stronger response requires a lower threshold of antigenic stimulation and replication of variants to which this response is directed is controlled by these CTL at a level below that needed to stimulate the weaker response. However, once viral heterogeneity occurs, the situation becomes much more complex and the pattern of CTL responses may become dependent upon fluctuations in variant sequences.

Two env-expressing recombinant vaccinia vectors were derived from patients VI-08 and VI-11 from early time points of infection and used as targets to evaluate early CTL responsiveness. Selective recognition of only one autologous env gene product was observed with pCTL quantitation of patient VI-08. This variability in CTL responsiveness to autologous and IIIB envs in CTL precursors quantitated in patient VI-08 over time may be representative of the dynamics of cellular immunity in the face of viral diversity. For example, at 6 months of age CTL precursors only recognized the env gene product derived at 20 days of age. Although there is a strong likelihood that sequences containing CTL epitopes were shared between the patient's autologous envs and IIIB, neither the 6 month autologous env nor IIIB env were recognized by pCTL from this time point. This strongly points to an immune response within patient VI-08 at 6 months of age which was

dominated by CTL specific for possibly one epitope. Lack of detection of pCTL against the autologous 6 month env at 6 months of age may have been due to an amino acid sequence change within this immunodominant epitope. This amino acid change could either result in complete loss of recognition of this epitope with a concommitant shift in peptide immunodominance within the host or the proliferation of a new pool of CTL precursors able to recognize this new epitope. If the latter case resulted, a shift in peptide immunodominance could still occur due to weaker recognition of this mutated epitope by this new pool ofCTL. A shift in immunodominance to an epitope(s) more commonly shared between both autologous envs and IIIB could explain the evolution by 19 months of age of group-specific env CTL responses.

Escape from CTL recognition within particular epitopes has been well-documented within HIV-1 infection (10-13, 116-123). However, it is frequently unclear as to whether loss of recognition of one epitope can result in complete abrogation of the CTL response to the entire gene product from which the epitope derived. The complete lack of recognition of autologous 6 month env by CTL in patient VI-08 at 6 months suggests that this can occur. Epitope mapping and variant sequencing are needed for confirmation, however, this situation is highly suggestive of complete escape of env from CTL recognition.

Precursor CTL of patient VI-11, in contrast, were able to recognize both autologous env gene products at three time points during the first year of age. Unlike patient VI-08, the amplified env genes used to generate the vaccinia recombinants from patient VI-11 displayed almost identical migration rates on heteroduplex mobility analysis (figure 4). This suggests that sequences within these two genes are very closely related. This is perhaps a result of amplifying env sequences from isolates taken at two closely apposed time intervals (1 month and 3 months of age), a result of selective pressures in viral culture for that particular env gene or simply a result of less variant diversity within isolates of patient VI-11 compared with patient VI-08. Recognition of both env gene products by

pCTL of patient VI-11 at all time points examined within the first year of life was therefore most likely due to a shared epitope(s).

It is important to point out that the method of in vitro stimulation used in these assays does not inherently favor the outgrowth of env CTL of any particular specificity. Assuming that all of the activated and memory CTL present within the PBMC have equal proliferative potential in response to nonantigen-specific stimulation, the proportion of pCTL directed against each target in the assay should be representative of that proportion in vivo. In this regard, in vitro stimulation without specific antigen provides a truer estimate of the dominant CTL response at that time point. Antigen-specific stimulation, however, can be important in providing appropriate conditions for the development of potent HIV-specific CTL lines (124) and detection of CTLs specific for epitopes recognized at low frequency (125). It is also required in order to demonstrate escape from CTL recognition with the greatest certainty.

G. Lack of cross-reactive pCTL in early infancy: immunologic naivete vs partial tolerance.

Although viral variants present soon after transmission appear to be genotypically homogeneous within the majority of adults and infants, early group-specific CTL responses undetectable in vertically infected infants are readily detected in adults. These CTL from adults have not been analyzed on a clonal level, therefore it is unclear whether group-specificity is due to cross-reactive pools of CTL or multiple pools of singly-reactive CTL, however, it seems logical to assume that cross-reactive CTL are partially responsible for the group-specific response because of viral homogeneity From split-well analyses of the pCTL measured against autologous and heterologous viral env products in patients VI-06, VI-05, VI-08 and VI-11 a pattern of CTL responsiveness emerges in which the primary

type-specific response appears to be mediated wholly by singly-reactive CTL. Patients VI-06 and VI-08 most clearly show this: Singly-reactive CTL precursors of patient VI-06 in cord blood recognized 1 day autologous env in the absence of recognition of IIIB env and singly reactive pCTL of patient VI-08 at 6 months recognized autologous 20 day env in the absence of recognition of autologous 6 month env or IIIB. Although patient VI-11 also had pCTL which only recognized autologous virus until 12 months of age, many of these were cross-reactive. This was probably due to recognition of a conserved immunodominant epitope within the 1 month and 3 month env sequences. What is of greater interest, however, is that as the infant matured, there was a transition in the nature of the reactivity of the population of pCTL responding to env. CTL precursors from early time points were predominantly singly-reactive resulting in type-specific CTL responses. Whereas, the majority of group-specific CTL at later time points were cross-reactive.

A parameter unique to early infant immunity which may allow for only singly-reactive, type-specific CTL responses could be the naive state of the infant immune system. Hypothetically, adults, whose immune systems have been primed by many previous viral infections, possess memory CTL to these viruses which can expand in response to HIV-1 infection. The proliferation of non HIV-specific memory CTL in response to HIV infection argues for the ability of the TCR on these cells to cross-react with HIV and heterologous virus. There may be a population of cells within these cross-reactive memory CTL which might also be more likely to elicit a group-specific HIV-1 response by their cross-reactive nature and may greatly contribute to the more vigorous CTL response seen in adults. Further support comes from the fact that the frequency of CTL precursors detected within early infant PBMC against autologous virus appears to be much lower than that seen in adults against heterologous virus (27-29, 32, 126).

Indeed, this phenomenon has been convincingly demonstrated in a murine model. Selin et al examined the clonal specificities and cross-reactivities of CTL in LCMV immune

mice later challenged with a heterologous viral infection such as Pichinde or vaccinia virus (127). Acute infection with the second virus resulted in expansion of memory CTLs specific for LCMV in which a large portion were cross-reactive with the second virus. Previous studies by this same group had also reported the expansion of a population of allospecific CTL during acute LCMV infection, many of which were also cross-reactive with LCMV (128). TCR cross-reactivity has also been demonstrated within the context of HIV-1. For example, some studies have demonstrated the ability of alloactivated CTL lines to also recognize regions of env (129, 130). Although Clerici et al (130) interpret their observation as a mechanism of HIV-1 pathogenesis through autoreactivity, it is more likely a natural phenomenon demonstrable with many different viruses. Additonally, Shirai et al (131) demonstrated TCR cross-reactivity to two different epitopes within env in a murine CTL line in which there was preferential Vβ usage.

Unlike adults, the immune system of young infants is not composed of memory pCTL poised to expand upon antigenic stimulation. Theoretically, anti-viral responses must arise primarily from naive CTL precursors. This might result in a delayed anti-HIV-1 CTL response which may be weaker than that seen in adults. Cross-reactive CTL may begin to appear as the infant's immune system accounters a greater complexity of HIV-1 viral variants as well as concommitant heterologous viral infections. Activated CTL derived from a smaller initial population may make lower overall contributions to the HIV-1 memory CTL pool due in part to elimination of many of these cells through apoptosis (132-136). Therefore, especially in the infant, the immune system may be constantly struggling to keep up with evolving quasispecies. This could be an important factor in rapid disease progression.

Alternatively, type-specific responses in early infancy in HIV-1 vertically infected infants may be due to partial tolerance. Infants become infected with HIV-1 at a time when

the cellular immune system is being vigorously edited and revised on the basis of its ability to discriminate between self and nonself both peripherally and within the thymic environment. 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 which have escaped 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. The expansion in epitope recognition may hypothetically result from a phenomenon observed in models of autoimmunity referred to as epitope spreading (137, 138) 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 months of age may evolve in this manner.

One might expect HIV-1-specific tolerance to occur in infants who have become infected in utero. However, infants infected both during the in utero and intrapartum periods display the same pattern of CTL responsiveness. Although it is not definitively known whether antigen encountered by infants during or shortly after birth is tolerizing, the protection of infants from vertical transmission of Hepatitis B Virus (HBV) by active vaccination at birth suggests that they are able to recognize an immunogen delivered during this period as non-self. However, it is also possible that antigen presentation may play a role in induction of tolerance as the HBV vaccine, being a non-infectious particle, may be presented to the immune system differently than HIV-1.

## H. Precursor Frequency Analysis of CTL against HLA-A2 restricted epitopes within PBMC of patients VI-06 and VI-08

The peptides 1923, 1930 and V3-1 all contain CTL epitope sequences defined in the literature as being HLA-A2 restricted (8, 87). All three of these sequences are based upon HIV-$1_{IIIB}$ env. Fortuitously, two of the patients, VI-06 and VI-08, possessed the A2 haplotype allowing for the possible mapping of the CTL response. PBMC of patient VI-06 at three months of age responded to two of these peptides: 1923 and V3-1. The precursor frequency against V3-1 was higher than that seen against the entire env at 6 or 12 months of age suggesting that this epitope was immunodominant for this patient. This was further supported by the degree of sequence change observed within this region between env clones from 1 day of age and those from 6 months of age. Moreover, precursor frequency measurements from PBMC at 14 months of age against this peptide from this patient fell below detection. It is important to note that this region is also part of the V3 loop and may therefore also be under selective pressures related to neutralizing antibody and/or viral tropism.

On the other hand, pCTL from patient VI-08, who also possessed the HLA-A2 allele and whose env clones from both 20 days and 6 months of age demonstrated high conservation of these three peptide sequences did not contain measurable CTL precursors at 6 months of age to IIIB env in which these peptide sequences reside. Furthermore, at 15 months of age, when pCTL to IIIB env were detected, the PBMC from this patient did not demononstrate pCTL to any of these peptides. Sequence variability within any of these peptides between the env clones derived at 20 days and those from 6 months of age was minimal, supporting the notion that these regions were not under selective pressure from an immune response.

Interestingly, a portion of the amino acid sequence of peptide V3-1, RPNNNTRKSI, was also reported by Safrit et al to be restricted by HLA-B7 and recognized by CTL clones derived from HLA-B7 HIV-1 infected patients (9). Moreover, patient VI-06 possesses the B7 haplotype, whereas patient VI-08 does not. The A2 restriction of this molecule is questionable as it does not contain a discernible A2 binding motif (139, 140). Therefore, an explanation for the lack of recognition of this env peptide by CTL precursors of patient VI-08 could be that this patient simply lacks the appropriate restricting class I allele.

Sequences of viral variants derived from patients recognizing the V3-1 peptide demonstrated little heterogeneity within this region, with complete conservation of the putative anchor residues (P at position 2 and I at position 10). Comparisons of sequences within laboratory virus strains IIIB, RF, MN and SF2 revealed this same conservation of anchor residues. Indeed, the same is true within the variants from both time points sequenced from patient VI-06. Only a sequence change of arginine to serine at position 9 (seen within a patient variant and HIV-1MN) could abrogate CTL recognition of this epitope but the effects of other amino acid changes on CTL recognition was not extensively examined. This region appeared to be relatively well conserved in patient-derived viral sequences from this study as well as in laboratory strains. It has been suggested that the V3-1 epitope would be useful for studying the cellular immune response to candidate HIV-1 vaccines. However, we have observed that this region within viral variants from patient VI-06 clearly evolved from relative homogeneity within the reported sequence to an amino acid sequence quite different outside of the anchor positions. Conservation of the anchor regions might still allow for binding of this peptide to HLA B7 however, changes outside of these positions might have resulted in escape from TCR recognition. Use of these mutated peptides in detection of CTL recognition by patient VI-06 is needed to confirm or deny this possibility.

A portion of peptide 1930, KLTPLCVTL, has been shown to bind with high affinity to HLA-A*0201 and be recognized by CTL clones derived from patients possessing this allele (95). Therefore, the recognition of this peptide by pCTL of patient VI-06 is probably in the context of A2. The lower precursor frequency to this peptide may be due to immunodominance of the B7 restricted response to peptide V3-1. The lack of recognition of IIIB env by pCTL of VI-08 is surprising considering that this patient's viral sequences demonstrate complete conservation of this IIIB peptide. These results suggest that caution must be taken in using sequencing analysis of viral variants to predict CTL reactivity. Clearly, other parameters need to be taken into account in addition to simply the presence or absence of a particular epitope within an individual with the appropriate class I haplotype. Specificities of peptide transporter systems and the cellular proteolytic machinery may also contribute to the selection of CTL epitopes (141, 142) as well as polymorphisms in HLA class I alleles. For example, Bergmann et al (143) reported that flanking residues can effect the presentation of HIV-1 epitopes within env to murine CTL. In addition, Buseyne et al (144) reported differential recognition of gag epitopes within CTL of patients who shared MHC alleles.

Lack of recognition of peptide 1923 in pCTL of patient VI-06 could be attributed to the choice of peptide used in the study. Previous studies have mapped A2 restricted recognition to the sequence LWVTVYYGVPVWKEATTTLFCA (8). However, finer mapping by Dupuis et al (95) demonstrate high binding affinity and CTL recognition of the amino terminal portion of this peptide: KLWVTVYYGV. The peptide used in detection of pCTL of patient VI-06 was VPVWKEATTTLFCASDAKAY which includes almost none of the smaller amino acid epitope. Again, proviral sequences of patient VI-08 contained this IIIB based peptide sequence in the absence of pCTL recognition.

I. ADCC antibodies directed against autologous and heterologous env protein.

Previous studies in our laboratory measuring ADCC antibodies in vertically infected infants demonstrated lack of detectable titers against heterologous env proteins following the loss of maternal antibody. Most of the antibody detected from birth to12 months was considered to be maternally derived and was usually group specific. Broliden et al (54) in a study designed to examine whether there was a correlation between ADCC antibody titers and vertical transmission, observed the same pattern in a total of 35 mother-infant pairs. ADCC antibody titers were measured against the IIIB and RF strains of HIV-1 through the first year of life in infected and uninfected infants and were compared with those of the mother's. In each case, the antibody titer from the mother matched that of the child through the first half year of life. Following this time point, however, little or no ADCC antibody was detected in these infants against HIV-1 laboratory strains. They concluded, from this study, that there was no correlation between maternal ADCC antibody titer and vertical transmission of HIV-1. In agreement with these studies, IIIB-env-specific ADCC antibody titers in plasma from patients VI-05, VI-08 and VI-11 were low to undetectable by six months of life and remained so out to 24 months of age which was the last time point examined in this study.

1. ADCC titers of patient VI-06 The pattern of ADCC antibody titers observed in patient VI-06 was unlike any described in Broliden et al (48) or from the three other infant's studied here. Equivalent titers to both IIIB and autologous 1 day env increased dramatically from two months to six months of age and was therefore attributed to strong antibody production by the infant within this early time point. The strong recognition of both heterologous and autologous envs is similar to the group-specificity of the CTL response in this patient in early infancy. Hypotheses concerning the possible

circumstances mediating the broadly reactive nature of this patient's immune response have been discussed previously.

2. ADCC titers of patient VI-05: IIIB specific ADCC antibody titers in the plasma of patient VI-05 present at 2months of age are close to that of autologous 2 month env. However, IIIB env ADCC antibody titers dropped below detection by 6 months of age while titers to autologous 2 month env remained the same. The loss of IIIB env ADCC antibody at 6 months of age is consistent with the loss of maternal antibody, however, the continued presence of antibody to autologous 2 month env is indicative of an active humoral immune response against that autologous virus. Patient VI-05 was a rapid progressor who died by 22 months of age. The drop in ADCC antibody titers from 6 to 20 months of age could be reflective of disease progression as some studies have reported a loss in ADCC responses with progression to AIDS (53) although there is lack of agreement on this subject. Ljunggren et al (145) observed that the presence of higher-titered ADCC antibody in infants greater than 6 months of age correlated with a better clinical prognosis. ADCC antibody titers to 6 month autologous env and IIIB were low to undetectable from 2 months to 20 months and 6 months to 20 months of age, respectively. This correlates with the lack of CTL recognition of 6 month autologous env at these time points. However IIIB env is recognized by CTL at later time points in the absence of ADCC antibody recognition. The 6 month autologous env appears to have escaped both CTL and ADCC recognition. It is possible that this was a minor variant within the patient and was not present at high enough levels to stimulate immunity. The discrepency between CTL and ADCC antibody recognition of IIIB env may be explained by epitope differences between these two immune responses. As this patient demonstrated only type-specific ADCC antibody responses subsequent to 2 months of age, the use of autolgous env to measure ADCC antibody in this patient demonstrated a pattern of antibody response which would have been missed if only IIIB responsiveness was assessed.

3. ADCC titers of patient VI-08: ADCC titers present at 2 months of age in plasma of patient VI-08 against both IIIB env and autologous 20 day env dropped to nearly undetectable levels by 6 months of age. This most likely represents a loss of maternal antibody. An increase in titer against autologous 20 day env from 6 to 24 months demonstrates the ability of this infant to generate type-specific ADCC antibody. Although the autologous 6 month env and IIIB env were recognized by CTL from 19 months of age, they failed to be recognized by ADCC antibody after 2 months of age. Again, different regions of the env gene recognized by CTL versus ADCC antibodies could account for this discrepancy. The lack of recognition of the autologous 6 month env may have resulted from mutations within the epitope(s) necessary for ADCC antibody production which allowed for escape from recognition.

4. ADCC titers of patient VI-11: Maternal ADCC antibody titers appear to be absent in patient VI-11 represented by the complete lack of detectable ADCC antibody to IIIB or autologous 1 month and 3 month envs. This observation may be explained by progressive disease in the patient's mother during the last trimester of pregnancy, however, no information regarding this is available. Patient generated group-specific ADCC antibody began to appear between 6 and 12 months of age, although ADCC titers were higher against autologous envs. The group-specificity of the antibody response at 12 months occurred at the time of detection of more broadly reactive CTL although the stronger recognition of autologous 3 month env is opposite of that seen by CTL at this time point.

5. In utero vs intrapartum timing of infection and induction of infant ADCC antibody responses: Patients VI-05 and VI-06 are documented as having been infected in utero. Because efficient placental transfer of antibody has been shown to occur within the last trimester, infection may have occurred either before or after the appearance of this antibody within the fetus. In either case, HIV-1 specific antibody titers may have been low

at the time of infection. Jelonek et al (146) have demonstrated that the ability of the offspring of HIV-1 env vaccinated mice to mount HIV-1 humoral responses upon vaccination was inhibited by the presence of maternal antibody. Reasons given for this inhibition include Fc-mediated inhibition, epitope masking and idiotypic perturbation of the infant's antibody response. Low titers of maternal antibody present at the time of in utero infection in infants VI-05 and VI-06 may have had less of an inhibitory effect resulting in earlier anti-HIV-1 infant antibody responses. Therefore, the ADCC antibody measured in these infants at two months of age could be made up in part by antibody produced by the infant. This could explain why a drop in antibody titer is not detected between two and six months in these two infants.

Intrapartum infection occurs following the completion of placental transfer of maternal antibody. These high titers of antibody may inhibit infant humoral responses prior to its clearance from the plasma. This could account for the lag in antibody production seen in patients VI-08 and VI-11. Although maternal antibody was not present in patient VI-11 that was capable of mediating ADCC, overall titers of anti-HIV-1 env antibodies transferred to this infant could still have been present. Positive anti-gp120, -gp160 and -gp41 banding patterns on Western blot by sera from this patient from birth to 3 months of age support this notion.

6. Type-specificity of infants ADCC responses: These data indicate that the infant is capable of generating ADCC antibody responses prior to a year of life. The specificity of these responses appears to differ from that of the CTL response in an intrapatient manner. For example, patient VI-05, who had group-specific CTL responses by 12 months of age displayed only type-specific ADCC responses at and beyond this time point. The same is true of patient VI-08 who had IIIB env responsiveness in the CTL response at a time point where there were no ADCC antibody titers to this env. Whereas, the opposite is true of patient VI-11. With this patient, ADCC titers were group specific from 6 to 24 months of

age while CTL responses were type-specific until 12 months of age. The early group-specific immunity observed in patient VI-06 represents the only situation where the specificity pattern correlated between ADCC antibody and CTL recognition. The V3 loop is a region of the env gene which can serve as an epitope for ADCC (50). In this study, CTL recognition of VI-06 was mapped to a region of V3. Group specific responsiveness in both CTL and ADCC antibody responses may be a result of a shared epitope in this case.

Env-specific antibodies detected by western blot in the first few months of life in patient VI-11 in the absence of measurable ADCC antibody titers suggests that there may be limited regions of env to which ADCC antibody are directed. This could be due in part to accessibility of epitopes because of folding and oligimerization of env on the cell surface. Synthetic peptides which can compete for binding of antibodies and truncated env expressing vaccinia vectors have been used to map the fine specificity of ADCC antibody recognition. The last 46 amino acids of the carboxyl end of gp120 to the midportion of gp41(147), two regions within the transmembrane of gp41(148, 149) and the V3 loop have all been reported as ADCC antibody epitopes (56). These have been mapped using adult sera shown to be broadly group-specific. In young infants, theoretically, epitope recognition may be more limited due to a the lack of primed B cells and memory CD4 helper cells. Limited recognition may also occur as a result of the young infant to recognize glycosylated regions of env. Infants lack T helper independent antibody production until approximately two years of life. These antibody responses are directed against polysaccharide antigen. Glycosylated regions of env may require a T helper independent antibody response of which these infants are incapable. Type-specific ADCC antibody responses may be more commonly observed in early infancy as a result.

7. In vivo antiviral ADCC in infants: The potential therapeutic role of ADCC antibody in HIV-1 infection in infants has not been clearly demonstrated. Jenkins, et al (71) demonstrated a defect in neonatal peripheral blood mononuclear cells mediating ADCC

cytotoxicity although direct NK cytolysis appeared to be intact. Therefore, although the appropriate antibody may be present, the ability of the effector cells to efficiently lyse infected cells through an ADCC mechanism may be impaired. Deleterious effects of ADCC antibody have been suggested as well. ADCC antibody may lyse uninfected CD4 cells coated with soluble gp120 (150).

## J. Implications for perinatal vaccination

Our results indicate that the young infant is capable of mounting HIV-1 specific immune responses. Vaccination of the infant at birth, particularly those exposed to HIV-1 in the intrapartum period, may be an effective strategy to interrupt transmission. One would be stimulating immune responses in the infant at a time when viral load is not only low, but relatively homogeneous. In light of our recent findings concerning the type-specificity of the immune response in early infants, the choice of viral strain for vaccination will be a complex issue. Had the intrapartum infected patients described in this study (VI-08 and VI-11) been vaccinated with a IIIB based env, CTL immunity in response to this env would probably not have cross-reacted with the transmitted virus, and would not have been protective. To manipulate CTL responses in vivo to respond dominantly to an invariant region of env one could use peptide-based vaccines. However, vaccines with such limited epitopes could be ineffective in a number of infants due to HLA restriction incompatibility. Administration of passive HIV-1 immunoglobulin at birth to prevent infection and dissemination accompanied by repeated boostings within the first year of life with varying heterologous strains could possibly overcome this problem. Neonatal delivery of passive and active immunization has been shown to be successful in protection from Hepatitis B infection. Successive immunizations would hypothetically be delivered to an immune system which would be acquiring a broader spectrum of activated and

memory pCTL as the infant aged from which broader env specific responses would be expected.

# REFERENCES

1. Bryant, M.L., and L. Ratner. 1992. Biology and molecular biology of human immunodeficiency virus. *J Pediatr Infect Dis* 11:390-400.

2. Delwart, E.L., E.G. Shpaer, G. Louwagie, F.E. McCutchen, M. Grez, H. Rubasamen-Waigmann, and J.I. Mullins. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. *Science* 262:1257-1261.

3. Balfe, P., P. Simmonds, C.A. Ludlam, B.J. O., and J.A. Leigh Brown. 1990. Concurrent evolution of human immunodeficiency virus type 1 in patients infected from the same source: rate of sequence change and low frequency of inactivating mutations. *J Virol* 64, no. 12:6221-6233.

4. Hahn, B.H., G.M. Shaw, M.E. Taylor, R.R. Redfield, P.D. Markham, S.Z. Salahuddin, F. Wong-Staal, R.C. Gallo, E.S. Parks, and W.P. Parks. 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. *Science* 232, no. 1548-1553.

5. Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, M.S. Saag, and G.M. Shaw. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373:117-122.

6. Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373:123-126.

7. Piatak, M.J., M.S. Saag, L.C. Yang, S.J. Clark, J.C. Kappes, K.C. Luk, B.H. Hahn, and J.D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259:1749-1754.

8. Dadaglio, G., A. Leroux, P. Langlade-Demoyen, E. Bahraoui, F. Traincard, R. Fisher, and F. Plata. 1991. Epitope recognition of conserved HIV envelope sequences by human cytotoxic T lymphocytes. *J Immunol* 147:2302-2309.

9. Safrit, J.T., A.Y. Lee, C.A. Andrews, and R.A. Koup. 1994. A region of the third variable loop of HIV-1 gp120 is recognized by HLA-B7 -restricted CTLs from two acute seroconversion patients. *J Immunol* 153:3822-3830.

10. Couillin, I., B. Culmann-Penciolelli, E. Gomard, J. Choppin, J. Levy, J. Guillet, and S. Saragosti. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the Human Immunodeficiency Virus 1 nef protein. *J Exp Med* 180:1129-1134.

11. Klenerman, P., S. Rowland-Jones, S. McAdam, B. Koppe, W. Rosenberg, D. Boyd, A. Edwards, P. Giangrande, R.E. Phillips, and A.J. McMichael. 1994. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 gag variants. *Nature* 369:403-410.

12. Phillips, R.E., S. Rowlands-Jones, D.F. Nixon, F.M. Gotch, J.P. Edwards, A.O. Ogunlesi, J.A. Rothbard, C.R.M. Bangham , C.R. Rizza, and A.J. McMichael. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 354:453-456.

13. Koenig, S., A.J. Conley, Y.A. Brewah, G.M. Jones, S. Leath, J.J. Boots, V. Davey, G. Pantaleo, J.F. Demarest, C. Carter, A. Wannebo, J.R. Yannelli, S.A. Rosenberg, and H.C. Lane. 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat Med* 1, no. 4:330-336.

14. Tsang, M.L., L.A. Evans, P. McQueen, L. Hurren, C. Byrne, R. Penny, B. Tindall, and D.A. Cooper. 1994. Neutralizing antibodies against sequential autologous

human immunodeficiency virus type 1 isolates after seroconversion. *J Infect Dis* 170:1141-1147.

15. Tersmette, M., R.A. Gruters, F. De Wolf, R.Y. de Goede, J.M.A. Lange, P.T. Schellekens, J. Goudsmit, H.G. Huisman, and F. Miedema. 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immune deficiency syndrome: Studies on sequential HIV isolates. *J Virol* 63:2118-2125.

16. Zhu, T., H. Mo, N. Wang, D.S. Nam, Y. Cao, R.A. Koup, and D.D. Ho. 1993. Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* 261:1179-1181.

17. Delwart, E.L., H.W. Sheppard, B.D. Walker, J. Goudsmit, and J.I. Mullins. 1994. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. *J Virol* 68:6672-6683.

18. Wolinsky, S.M., C.M. Wike, B.T.M. Korber, C. Hutto, W. Parks, L.L. Rosenblum, K.J. Kunstman, M.R. Furtado, and J. L.Munoz. 1992. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* 255:1134-1137.

19. Buchbinder, S.P., M.H. Katz, N.A. Hessol, P.M. O'Malley, and S.D. Holmberg. 1994. Longterm HIV-1 infection without immunologic progression. *AIDS* 8:1123-1128.

20. Rutherford, G., A. Lifson, N. Hessol, W. Darrow, P. OMalley, S. Buchbinder, J. Barnhart, T. Bodecker, L. Cannon, L. Doll, S. Holmberg, J. Harrison, M. Rogers, D. Werdegar, and H. Jaffe. 1990. Course of HIV-1 infection in a cohort of homosexual and bisexual men: an 11 year follow up study. *BMJ* 301:1183-1188.

21. Kirchhoff, F., T. Greenough, D. Brettler, J. Sullivan, and R. Desrosiers. 1995. Absence of intact nef sequences in a long-term, nonprogressing survivor of HIV-1 infection. *N Engl J Med* 332:228-232.

22. Greenough, T.C., M. Somasundaran, D.B. Brettler, R.M. Hesselton, A. Alimenti, F. Kirchhoff, D. Panicali, and J.L. Sullivan. 1994. Normal immune function and inability to isolate virus in culture in an individual with long-term human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses* 10, no. 4:395-403.

23. Walker, B.D., S. Chakrabarti, B. Moss, T.J. Paradis, T. Flynn, A.G. Durno, R.S. Blumberg, J.C. Kaplan, M.S. Hirsch, and R.T. Schooley. 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* 328:345-348.

24. Koup, R.A., J.L. Sullivan, P.H. Levine, D. Brettler, A. Mahr, G. Mazzara, S. McKenzie, and D. Panicali. 1989. Detection of major histocompatibility complex class I restricted HIV-specific cytotoxic T lymphocytes in the blood of infected hemophiliacs. *Blood* 73:1909-1914.

25. Rinaldo, A.R., L.A. Beltz, X. Huang, P. Gupta, Z. Fan, and D.J. Torpey. 1995. Anti-HIV type 1 cytotoxic T lymphocyte effector activity and disease progression in the first 8 years of HIV type 1 infection of homosexual men. *AIDS Res Hum Retroviruses* 11, no. 4:481-489.

26. Klein, M.R., C.A. van Baalen, A.M. Holwerda, S.R. Kerkhof Garde, R.J. Bende, I.P.M. Keet, J.M. Eeftinck-Schattenkerk, M.E. Osterhaus, H. Schuitemaker, and F. Miedema. 1995. Kinetics of gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* 181:1365-1372.

27. Moss, P.A., S.L. Rowland-Jones, P.M. Frodsham, S. McAdam, P. Giangrande, A.J. McMichael, and J.I. Bell. 1995. Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors. *Proc Natl Acad Sci, USA* 92:5773-5777.

28. Rinaldo, C., X. Huang, Z. Fan, M. Ding, L. Beltz, A. Logar, D. Panicali, G. Mazzara, J. Liebmann, M. Cottrill, and P. Gupta. 1995. High levels of anti-human

immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1 infected long-term nonprogressors. *J Virol* 69, no. 9:5838-5842.

29. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D.D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68:4650-4655.

30. Carmichael, A., X. Jin, P. Sissons, and L. Borysiewicz. 1993. Quantitiative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein Barr virus in late disease. *J Exp Med* 177:249-256.

31. Gotch, F.M., D.F. Nixon, N. Alp, A.J. McMichael, and L.K. Borysiewicz. 1990. High frequency of memory and effector gag specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int Immunol* 2:707-711.

32. Koup, R.A., C.A. Pikora, K. Luzuriaga, D.B. Brettler, E.S. Day, G.P. Mazzara, and J.L. Sullivan. 1991. Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: in vitro quantitation of effector cell populations with p17 and p24 specificities. *J Exp Med* 174, no. 6:1593-600.

33. Johnson RP, T.A., Yang L, Mazzara G, Panicali D, Buchanan T, Walker BD. 1991. HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes: fine specificity of the gag-specific responses by using unstimulated peripheral blood mononuclear cells and cloned effector cells. *J Immunol* 147:1512.

34. Lieberman J, F.J., Kuo M, Earl P, Moss B, Skolnik PR. 1992. Cytotoxic T lymphocytes from HIV-1 seropositive individuals recognize immunodominant epitopes in gp160 and reverse transcriptase. *J Immun* 148, no. 9:2738-2747.

35. Walker, B.D., C. Flexner, T.J. Paradis, T.C. Fuller, M.S. Hirsch, R.T. Schooley, and B. Moss. 1988. HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. *Science* 240:64-66.

36. Borrow, P., H. Lewicki, B.H. Hahn, G.M. Shaw, and M.B. Oldstone. 1994. Virus specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Viology* 68:6103-6110.

37. Kannagi, M., T. Masuda, T. Hattori, K. Nasu, N. Yamamoto, and S. Harada. 1990. Interference with Human Immunodeficiency Virus (HIV) replication by CD8+ T cells in peripheral blood leukocytes of asymptomatic HIV carriers in vitro. *J Virol* 64, no. 7:3399-3406.

38. Tsubota, H., C.I. Lord, D.I. Watkins, C. Morimoto, and N. Letvin. 1989. A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrom virus replication in peripheral blood lymphocytes. *J Exp Med* 169:1421.

39. Walker, C.M., D.J. Moody, D.P. Stites, and J.A. Levy. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234:1563.

40. Koenig, S., B. van Kuyk, G. Jones, B. Torbett, D. Mosier, Y.A. Brewah, S. Leath, V.J. Davey, J. Yanelli, S. Rosenberg, A.S. Fauci, and H.C. Lane. 1992. Adoptive transfer of a nef-specific CTL clone into hu-PBL-SCID mice and an HIV-infected patient. VIII international Conference on AIDS/III STD World Conference, Amsterdam, Netherlands.

41. Javerhavian, K., A.J. Langlois, G.J. LaRosa, A.T. Profy, D.P. Bolognesi, W.C. Herlihy, S.D. Putney, and T.J. Matthews. 1990. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. *Science* 250:1590.

42. Javaherian, K., A.J. Langlois, C. McDanal, K.L. Ross, L.I. Eckler, C.L. Jellis, A.T. Profy, J.R. Rusche, D.P. Bolognesi, S.D. Putney, and T.J. Matthews. 1989.

Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc Natl Acad Sci USA* 86:6768.

43. Profy, A.T., P.A. Salinas, L.I. Eckler, N.M. Dunlop, P.L. Nara, and S.D. Putney. 1990. Epitopes recognized by the neutralizing antibodies of an HIV-1-infected individual. *J Immunol* 144:4641.

44. Sun, N.C., D.D. Ho, C.R. Sun, R.S. Liou, W. Gordon, M.S. Fung, X.L. Li, R.C. Ting, T.H. Lee, N.T. Chang, and T.W. Chang. 1989. Generation and characterization of monoclonal antibodies to the putative CD4-binding domain of human immunodeficiency virus type gp120. *J Virol* 65:3579.

45. Lasky, L.A., G. Nakamura, D.H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P.W. Berman, T. Gregory, and D.J. Capon. 1987. Delineation of a region of the human immunodeficiency type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* 50(6):975.

46. Fung, M.S., C.R. Sun, W.L. Gordon, R.S. Liou, T.W. Chang, W.N. Sun, E.C. Caar, and D.D. Ho. 1992. Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type 1 gp120. *J Virol* 66:848.

47. Kang, C.Y., P. Nara, S. Chamat, V. Caralli, T. Ryskamp, N. Haigwood, R. Newman, and H. Kohler. 1991. Evidence for non-V3 specific neutralizing antibodies that interfere with gp120/CD4 binding in human immunodeficiency 1-infected humans. *Proc Natl Acad Sci USA* 88:6171.

48. Ho, D.D., J.A. McKeating, X.L. Li, T. Moudgil, E.S. Daar, N.C. Sun, and J.E. Robinson. 1991. Conformational epitope on gp120 important for CD4 binding and HIV-1 neutralization identified by a human monoclonal antibody. *J Virol* 65:489.

49. Thali, M., U. Olshevsky, C. Furman, D. Gabuzda, M. Posner, and J. Sodroski. 1991. Characterization of a discontinuous human immunodeficiency virus type 1 gp 120

epitope recognized by a broadly reactive neutralizing human monoclonal antibody. *J Virol* 65, no. 848.

50. Emini, E.A., W.A. Schleif, J.H. Nunberg, A.J. Conley, Y. Eda, S. Tokiyoshi, S.D. Putney, S. Matsushita, K.E. Cobb, and C.M. Jett. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* 355:728-730.

51. Albert, J., B. Abrahamsson, and K. Nagy. 1991. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. *AIDS* 4:107-112.

52. Koup, R.A., C.A. Pikora, G. Mazzara, D. Panicali, and J.L. Sullivan. 1991. Broadly reactive antibody-dependent cellular cytotoxic response to HIV-1 envelope glycoproteins precedes broad neutralizing response in human infection. *Viral Immunol* 4, no. 4:215-23.

53. Ljunggren, K., B. Bottiger, G. Biberfeld, A. Karlson, E. Fenyo, and M. Jondal. 1987. Antibody-dependent cellular cytotoxicity-inducing antibodies against human immunodeficiency virus: Presence at different clinical stages. *J Immunol* 139, no. 7:2263-2267.

54. Broliden, K., E. Sievers, P.A. Tovo, V. Moschese, G. Scarlatti, P.A. Broliden, C. Fundaro, and P. Rossi. 1993. Antibody-dependent cellular cytotoxicity and neutralizing activity in sera of HIV-1-infected mothers and their children. *Clin Exp Immunol* 93:56-64.

55. Zeigner, U.H.M., I. Frank, A. Bernatowicz, S.E. Starr, and S. H. 1992. Antibody-dependent cellular cytotoxicity (ADCC) is directed against immunodominant epitopes of the envelope proteins of human immunodeficiency virus 1 (HIV-1). *Viral Immunol* 5, no. 4:273-281.

56. Vogel, T., R. Kurth, and S. Norley. 1994. The majority of neutralizing Abs in HIV-1-infected patients recognize linear V3 loop sequences. *J Immunol* 153:1895-1904.

57. Kohl, S., and L.S. Loo. 1982. Protection of neonatal mice against herpes simplex virus infection: probable in vivo antibody-dependent cellular cytotoxicity. *J Immunol* 129:370-376.

58. Zisman-Rager, B., and A.C. Allison. 1976. Mechanism of immunologic resistance to herpes simplex virus 1 (HSV-1) infection. *J Immunol* 116:35-40.

59. Lyerly, H.K., D.S. Tyler, C.L. Nastala, and K.J. Weinhold. 1989. Anti-HIV-1 ADCC, eds. S.D. Putney and D.P. Bolognesi. Decker, New York.

60. Rogers, M.F., M.B. Caldwell, M.L. Gwinn, and R.J. Simonds. 1994. Epidemiology of pediatric Human Immunodeficiency virus infection in the United States. *Acta Paediatr Suppl* 400:5-7.

61. Delfraissy, J.F., S. Blanche, C. Rouzioux, and M.J. Mayaux. 1992. Perinatal HIV transmission facts and controversies. *Immunodef Rev* 3:305-327.

62. Schwartz, D.A., and A.J. Nahmias. 1991. Human Immunodeficiency virus and the placenta. *Ann Clin Lab Sci* 21, no. 4:264-274.

63. Wilfert CM, W.C., Luzuriaga K, Epstein L. 1994. Pathogenesis of pediatric human immunodeficiency virus type 1 infection. *J Infect Dis* 170, no. 2:286-92.

64. Luzuriaga, K., R.A. Koup, C.A. Pikora, D.B. Brettler, and J.L. Sullivan. 1991. Deficient human immunodeficiency virus type 1-specific cytotoxic T cell responses in vertically infected children. *J Pediatr* 119, no. 2:230-6.

65. Buseyne, F., S. Blanche, D. Schmitt, C. Griscelli, and Y. Riviere. 1993. Detection of HIV-specific cell-mediated cytotoxicity in the peripheral blood from infected children. *J Immunol* 150:3569.

66. Froebel KS, A.M., Mok JYQ, Hayley J, Arnott M, Peutherer JF. 1994. Cytotoxic T Lymphocyte Activity in Children Infected with HIV. *AIDS Res Hum Retroviruses* 10, Supplement 2:S83-S88.

67. McFarland, E.J., P.A. Harding, D. Luckey, B. Conway, R.K. Young, and D.R. Kuritzkes. 1994. High frequency of gag- and env-specific cytotoxic T lymphocyte precursors in children with vertically-acquired human immunodeficiency virus type 1 infection. *J Infect Dis* 170:766-774.

68. Luzuriaga, K., D. Holmes, A. Hereema, J. Wong, D. Panicali, and J. Sullivan. 1995. HIV-1-Specific cytotoxic T lymphocyte responses in the first year of life. *J Immunol* 154:433-443.

69. Husson, R.N., Y. Lan, E. Kojima, D. Venzon, H. Mitsuya, and K. McIntosh. 1995. Vertical transmission of human immunodeficiency virus type 1: Autologous neutralizing antibody, virus load, and virus phenotype. *J Pediatr* 126:865-71.

70. Scarlatti, G., J. Albert, P. Rossi, V. Hodara, P. Biraghi, L. Muggiasca, and E.M. Fenyo. 1993. Mother-to-child transmission of Human Immunodeficiency Virus Type 1: Correlation with neutralizing antibodies against primary isolates. *J Infect Dis* 168:207-210.

71. Jenkins, M., J. Mills, and S. Kohl. 1993. Natural killer cytotoxicity and antibody-dependent cellular cytotoxicity of human immune deficiency virus-infected cells by leukocytes from human neonates and adults. *Pediatr Res* 33(5):469-474.

72. Ahmed, R., A. salmi, L.D. Butler, J. Chiller, and M.B.A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160:521-540.

73. von Herrath, M.G., J. Dockter, M. Nerenberg, J.E. Gairin, and M.B.A. Oldstone. 1994. Thymic selection and adaptability of cytotoxic T lymphocyte responses in transgenic mice expressing a viral protein in the thymus. *J Exp Med* 180:1901-1910.

74. Valentin, H., M. Nugeyre, F. Vuillier, L. Boumsell, M. Schmid, F. Barre-Sinoussi, and R.A. Pereira. 1994. Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro. *J Virol* 68, no. 5:3041-3050.

75. Burchett, S.K., L. Corey, K.M. Mohan, J. Westall, R. Ashley, and C. Wilson. 1992. Diminished interferon gamma and lymphocyte proliferation in neonatal and postpartum primary herpes simplex virus infection. *J Infect Dis* 165, no. 5:813-818.

76. Bryson, Y.J., H.S. Winter, S.E. Gard, T.J. Fischer, and E.R. Stiehm. 1980. Deficiency of immune interferon production by leukocytes of normal newborns. *Cell Immunol* 55:191-200.

77. Wilson, C.B., J. Westall, L. Johnston, D.B. Lewis, S.K. Dower, and A.R. Alpert. 1986. Decreased production of interferon gamma by human neonatal cells: intrinsic and regulatory deficiencies. *J Clin Invest* 77:860-867.

78. Lewis, D.B., A. Larsen, and C.B. Wilson. 1986. Reduced interferon-gamma mRNA levels in human neonates. *J Exp Med* 163:1018-1023.

79. Budd, R.C., J.C. Cerottini, and H.R. MacDonald. 1987. Selectively increased production of interferon-gamma by subsets of Lyt-2+ and P3T4+ T cells identified by expression of Pgp-1. *J Immunol* 138:3583-3586.

80. Fuleihan, R., D. Ahern, and R. Geha. 1994. Decreased expression of the ligand for CD40 in newborn lymphocytes. *Eur J Immunol* 24:1925-1928.

81. Harris, D.T., M.J. Schumacher, J. Locascio, F.J. Besencon, G.B. Olson, D. DeLuca, L. Shenker, J. Bard, and E.A. Boyse. 1992. Phenotypic and functional

immaturity of human umbilical cord blood T lymphocytes. *Proc Natl Acad Sci, USA* 89:10006-10010.

82. Stern, D.A., M.J. Hicks, R.D. Martinez, C.J. Holberg, A.L. Wright, J. Pinnas, M. Halonen, and L.M. Taussig. 1992. Lymphocyte subpopulation number and function in infancy. *Dev Immunol* 2:175-179.

83. Weiss, R.A., P.R. Clapham, and R. Cheingsong-Popov. 1985. Nautralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. *Nature* 316:69-72.

84. Robert-Guroff, M., M. Brown, and R.C. Gallo. 1985. HTLV-III neutralizing antibodies in patients with AIDS and AIDS-related complex. *Nature* 316:72-74.

85. Bryson, Y.J., K. Luzuriaga, J.L. Sullivan, and D.W. Wara. 1992. Proposed definitions for in utero versus intrapartum transmission of HIV-1 [letter]. *New Engl J Med* 327, no. 17:1246-7.

86. Myers, G., B. Korber, S. Wain-Hobson, K.-T. Jeang, L. Henderson, and G.N. Pavlakis, editors. 1994. Human Retroviruses and AIDS, 1994. Theoretical Biology and Biophysics, Los Alamos, New Mexico.

87. Clerici M, L.D., Zajac RA, Boswell RN, Gebel HM, Takahashi H, Berzofsky JA, Shearer GM. 1991. Detection of cytotoxic T lymphocytes specific for synthetic peptides of gp160 in HIV-seropositive individuals. *J Immunol* 146, no. 7:2214-2219.

88. Mazzara, G., A. Destree, and A. Mahr. 1993. Generation and analysis of vaccinia virus recombinants. *Methods Enzymol* 217:557-581.

89. Kuiken, C.L., J.J. DeJong, E. Baan, W. Keulen, M. Tersmette, and J. Goudsmit. 1992. Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. *J Virol* 66:4622-4627.

90. Kusumi, K., B. Conway, S. Cunningham, A. Berson, C. Evans, A. Iversen, D. Colvin, M. Gallo, S. Coutre, E. Shpaer, D. Faulkner, A. DeRonde, S. Volkman, C. Williams, M. Hirsch, and J. Mullins. 1992. Human immunodeficiency virus type 1 envelope gene structure and diversity in vivo and after cocultivation in vitro. *J Virol* 66:875-885.

91. Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Asjo, and S. Wain-Hobson. 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* 58:901-910.

92. Safrit JT, K.R. 1995. The immunology of primary HIV infection: which immune responses control HIV replication? *Curr Opin Immunol* 7:456-461.

93. Nixon AF, M.A. 1991. Cytotoxic T-cell recognition of HIV proteins and peptides. *AIDS* 5:1049-1059.

94. Kalams SA, W.B. 1994. The cytotoxic T-lymphocyte response in HIV-1 infection. *Clin Lab Med* 14, no. 2:271-299.

95. Dupuis M, K.S., Merigan TC. 1995. Characterization of *HLA-A*0201* - restricted cytotoxic T cell epitopes in conserved regions of the HIV Type 1 gp160 protein. *J Immunol* 155:2232-2239.

96. Safrit JT, A.C., Zhu T, Ho DD, Koup RA. 1994. Characterization of Human Immunodeficiency Virus Type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. *J Exp Med* 179:463-472.

97. Johnson, R.P., A. Trocha, T.M. Buchanan, and B.D. Walker. 1992. Identification of overlapping HLA class I-restricted cytotoxic T cell epitopes in a conserved region of the human immunodeficiency virus type 1 env glycoprotein: definition of minimum epitopes and analysis of the effects of sequence variation. *J Exp Med* 175:961-971.

98. Walker, B.D., K. Flexner, K. Birch-Limberger, L. Fisher, T. Paradis, A. Aldovini, R. Young, B. Moss, and R.T. Schooley. 1989. Long-term culture and fine specificity of human cytotoxic T lymphocyte clones reactive with human immunodeficiency type 1. *Proc Natl Acad Sci* 86:9514-9518.

99. Luzuriaga, K., P. McQuilken, A. Alimenti, M. Somasundaran, R. Hesselton, and J.L. Sullivan. 1993. Early viremia and immune responses in vertical human immunodeficiency virus type 1 infection. *J Infect Dis* 167, no. 5:1008-13.

100. Cheynier, R., P. Langlade-Demoyen, M.R. Marescot, S. Blanche, G. Blondin, S. Wain-Hobson, C. Griscelli, E. Vilmer, and F. Plata. 1992. Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1 infected mothers. *Eur J Immunol* 22:2211-2217.

101. Zhang LQ, M.P., Cleland A, Holmes EC, Leigh Brown AJ, Simmonds P. 1993. Selection for Specific Sequences in the External Envelope Protein of Human Immunodeficiency Virus Type I upon Primary Infection. *J Virol* 67, no. 6:3345-3356.

102. Tuofu Z, W.N., Carr A, Wolinsky S, Ho D. 1995. Evidence for coinfection by multiple strains of Human Immunodeficiency Virus Type 1 Subtype B in an acute seroconverter. *J Virol* 69, no. 2:1324-1327.

103. Riviere, Y., F. Tanneau-Salvadori, A. Regnault, O. Lopez, P. Sansonetti, B. Guy, M.P. Kieny, J.J. Fournel, and L. Montagnier. 1989. Human immunodeficiency virus-specific cytotoxic responses of seropositive individuals:distinct types of effector cells mediate killing of targets expressing gag and env. *J Virol* 63: 2270-2277.

104. McChesney, M., F.Tanneau, A. Regnault, P. Sansonetti, L. Montagnier, M.P. Kieny, and Y. Riviere. 1990. Detection of primary cytotoxic T lymphocytes specific for the env glycoprotein of HIV-1 by deletion of the env amino-terminal signal sequence. *Eur J Immunol* 20:215-220.

105. Townsend ARM, R.J., Gotch FM, Bahadur G, Wraith D, McMichael AJ. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959.

106. Whitton JL, G.J., Lewicki H, Tishon A, Oldstone MBA. 1988. Molecular analysis of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J Virol* 63:4303.

107. Whitton JL, G.J., Lewicki H, Tishon A, Oldstone MBA. 1988. Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis. *J Virol* 62:687.

108. Klavinskis LS, W.J., Oldstone MBA. 1989. Molecularly engineered vaccine which expresses an immunodominant T-cell epitope induces cytotoxic T lymphocytes that confer protection from lethal virus infection. *J Virol* 63:4311.

109. Braciale TJ, S.M., Morrison LA, Kittlesen DJ, Braciale VL. 1989. Class I major histocompatibility complex-restricted cytolytic T lymphocytes recognize a limited number of sites on the influenza hemaglutinin. *Proc Nat Acad Sci, USA* 86:277.

110. Hosmalin A, C.M., Houghten R, Pendleton CD, Flexner C, Lucey DR, Moss B, Germain RN, Shearer GM, Berzofsky JA. 1990. An epitope in human immunodeficiency virus 1 reverse transcriptase recognized by both mouse and human cytotoxic T lymphocytes. *Proc Nat Acad Sci, USA* 87:2344.

111. Takahashi H, C.J., Hosmalin A, Cease KB, Houghten R, Cornette JL, DeLisi C, Moss B, Germain RN, Berzofsky JA. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc Nat Acad Sci, USA* 85:3105-3109.

112. Takahashi H, M.S., Putney SD, Houghten R, Moss B, Germain RN, Berzofsky JA. 1989. A single amino acid interchange yields reciprocal CTL specificities for HIV-1 gp160. *Science* 246:118.

113. Takahashi H, H.R., Putney SD, Margulies DH, Moss B, Germain RN, Berzofsky JA. 1989. Structural requirements for class I MHC molecule-mediated antigen presentation and cytotoxic T cell recognition of an immunodominant determinant of the human immunodeficiency virus envelope protein. *J Exp Med* 170:2023.

114. Cease KB, M.H., Cornette JL, Putney SD, Robey WG, Ouyang C, Streicher HZ, Fischinger PJ, Gallo RC, DeLisi C, Berzofsky JA. 1987. Helper T-cell antigenic site identification in the acquired immunodeficiency syndrome virus gp120 envelope protein and induction of immunity in mice to the native protein using a 16-residue synthetic peptide. *Proc Nat Acad Sci, USA* 84:4249-4253.

115. Nowak MA, M.R., Phillips RE, Rowland-Jones S, Lalloo DG, McAdam S, Kienerman P, Koppe B, Sigmund K, Bangham CRM, McMichael AJ. 1995. Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* 375:606-611.

116. Koup, R.A. 1994. Virus escape from CTL recognition. *J Exp Med* 180:779-782.

117. Allen, P.M., and R.M. Zinkernagel. 1994. Promethean viruses? *Nature* 369:355-356.

118. Bergmann, C.C., L. Tong, R.V. Cua, J.L. Sensintaffar, and S.A. Stohlman. 1994. Cytotoxic T cell repertoire selection. *J Immunol* 152:5603-5612.

119. Phillips, R.E., and A.J. McMichael. 1993. How does the HIV escape cytotoxic T cell immunity? *Chem Immunol* 56:150-164.

120. Rowland-Jones, S., R.E. Phillips, D.F. Nixon, F.M. Gotch, J.P. Edwards, A.O. Ogunlesi, J.G. Elvin, J.A. Rothbard, C.R.M. Bangham, C.R. Rizza, and A.J. McMichael. 1992. Human Immunodeficiency Virus variants that escape cytotoxic T-cell recognition. *AIDS Res Hum Retroviruses* 8, no. 8:1353-1354.

121. Franco, A., C. Ferrari, A. Sette, and F.V. Chisari. 1995. Viral mutations, TCR antagonism and escape from the immune response. *Curr Opin Immunol* 7:524-531.

122. Couillin, I., F. Connan, B. Culmann-Penciolelli, E. Gomard, J. Guillet, and J. Choppin. 1995. HLA-dependent variations in human immunodeficiency virus nef protein alter peptide/HLA binding. *Eur J Immunol* 25:728-732.

123. Klenerman, P., U. Meier, R.E. Phillips, and A.J. McMichael. 1995. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. *Eur J Immunol* 25:1927-1931.

124. Lieberman, J., J.A. Fabry, P. Shankar, L. Beckett, and P.R. Skolnik. 1995. Ex Vivo expansion of HIV Type 1-specific cytolytic T cells from Type 1-seropositive subjects. *AIDS Res Hum Retroviruses* 11, no. 2:257-271.

125. Shankar, P., J. Fabry, and J. Lieberman. 1995. A simple method to selectively expand HIV-1 cytotoxic T lymphocytes in vitro. *Immunol Invest* 24, no. 3:489-497.

126. Lamhamedi-Charradi, S., B. Culmann-Penciolelli, B. Guy, M. Kieny, F. Dreyfus, A. Saimot, D. Sereni, D. Sicard, J. Levy, and E. Gomard. 1992. Qualitative and quantitative analysis of human cytotoxic T-lymphocyte responses to HIV-1 proteins. *AIDS* 6:1249-1258.

127. Selin, L.K., S.R. Nahill, and R.M. Welsh. 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous virus. *J Exp Med* 179:1933-1943.

128. Nahill, S.R., and R.M. Welsh. 1993. High frequency of cross-reactive cytotoxic T lymphocytes elicited during the virus-induced polyclonal cytotoxic T lymphocyte response. *J Exp Med* 177:317-326.

129. Atassi, H., and A.M. Z. 1992. HIV envelope protein is recognized as an alloantigen by human DR-specific alloreactive T cells. *Hum Immunol* 34:31-38.

130. Clerici, M., G. Shearer, E.F. Hounsell, B. Jameson, J. Habeshaw, and A.G. Dalgleish. 1993. Alloactivated cytotoxic T cells recognize the carboxy-terminal domain of

human immunodeficiency virus-1 gp120 envelope glycoprotein. *Eur J Immunol* 23, no. 2022-2025.

131. Shirai, M., M.S. Vacchio, R.J. Hodes, and J.A. Berzofsky. 1993. Preferential Vβ usage by cytotoxic T cells cross-reactive between two epitopes of HIV-1 gp160 and degenerate in class I MHC restriction. *J Immunol* 151, no. 4:2283-2295.

132. Razvi, E.S., and R.M. Welsh. 1993. Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency. *J Virol* 67, no. 10:5754-5765.

133. Razvi, E., and R.M. Welsh. 1994. Apoptosis in viral infections. *Adv virus res* 45:1-60.

134. Razvi, E.S., Z. Jiang, B.A. Woda, and R. Welsh. 1995. Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. *Am J Path* 147, no. 1:79-91.

135. Russell, J.H. 1995. Activation-induced death of mature T cells in the regulation of immune responses. *Curr Opin Immunol* 7:382-388.

136. Selin, L.K., and R.M. Welsh. 1994. Specificity and editing by apoptosis of virus-induced cytotoxic T lymphocytes. *Curr Opin Immunol* 6:553-559.

137. Lehmann, P.V., T. Forsthuber, A. Miller, and E.E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155-157.

138. James, J.A., T. Gross, R.H. Scofield, and J.B. Harley. 1995. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce sliceosome autoimmunity. *J Exp Med* 181:453-461.

139. Rammensee, H. 1995. Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin Immunol* 7:85-96.

140. Parker, K.C., M.A. Bednarek, L.K. Hull, U. Utz, B. Cunningham, H.J. Zweerink, W.E. Biddison, and J.E. Coligan. 1992. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J Immunol* 149, no. 11:3580-3587.

141. Neefjes, J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* 261:769-771.

142. Del Val, M., H. Schlicht, T. Ruppert, M. Reddehase, and U. Kozinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecule depends on its neighvoring residues in the protein. *Cell* 66:1145-1153.

143. Bergmann, C.C., L. Tong, R. Cua, J. Sensintaffar, and S. Stohlman. 1994. Differential effects of flanking residues on presentation of epitopes from chimeric peptides. *J Virol* 68, no. 8:5306-5310.

144. Buseyne, F., G. Janvier, B. Fleury, D. Schmidt, and Y. Riviere. 1994. Multispecific and heterogeneous recognition of the gag protein by cytotoxic T lymphocytes (CTL) from HIV-infected patients: factors other than the MHC control the epitopic specificities. *Clin Exp Immunol* 97:353-360.

145. Ljunggren, K., K. Moschese, and P.A. Broliden. 1990. Antibodies mediating cellular cytotoxicity and neutralization correlate with a better clinical stage in children born to human immunodeficiency virus-infected mothers. *J Infect Dis* 161:198-202.

146. Jelonek, M.T., L.M. Maskrey, K.S. Steimer, B.J. Potts, K.W. Higgins, and M.A. Keller. 1995. Inhibition of the offspring anti-recombinant gp120 antibody response to a human immunodeficiency virus vaccine by maternal immunization in a murine model. *J Infect Dis* 172:539-542.

147. Kenealy, W., D. Reed, R. Cybulski, D. Tribe, P. Taylor, C. Stevens, T. Matthews, and S. Petteway. 1987. Analysis of human serum antibodies to human

immunodeficiency virus (HIV) using recombinant env and gag antigens. *AIDS Res Hum Retroviruses* 3:95.

148. Evans, L.A., G. Thomson-Honnevier, K. Steimer, E. Paoletti, M.E. Perkus, A. Hollander, and J.A. Levy. 1989. Antibody-dependent cellular cytotoxicity is directed against both the gp120 and gp41 envelope proteins of HIV. *AIDS* 3:273.

149. Blumberg, R.S., T. Paradis, K.L. Hartshorn, M. Vogt, D.D. Ho, M.S. Hirsch, J. Leban, V.L. Sato, and R.T. Schooley. 1987. Antibody-dependent cell mediated cytotoxicity against cells infected with human immunodeficiency virus. *J Infect Dis* 156:878.

150. Weinhold, K.J., H.K. Lyerly, T.J. Matthews, S.S. Tyler, P.M. Ahearne, K.C. Stine, A.J. Langlois, D.T. Durack, and D.P. Bolognesi. 1988. Cellular anti-gp120 reactivities in HIV-1 seropositive individuals. *Lancet* 1:902-904.