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*J Immunol* 1999; 162:4796-4800; 
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Persistent HIV-1-Specific CTL Clonal Expansion Despite High Viral Burden Post In Utero HIV-1 Infection

Christian Brander,* Philip J. R. Goulder,* Katherine Luzuriaga,† Otto O. Yang* Kelly E. Hartman,* Norman G. Jones,* Bruce D. Walker,* and Spyros A. Kalams*

To address the issue of clonal exhaustion in humans, we monitored HLA class I-restricted, epitope-specific CTL responses in an in utero HIV-1-infected infant from 3 mo through 5 years of age. Serial functional CTL precursor assays demonstrated persistent, vigorous, and broadly directed HIV-1 specific CTL activity with a dominant response against an epitope in HIV-1 Gag-p17 (SLYNTVATL, aa 77–85). A clonal CTL response directed against the immunodominant, HLA-A*0201-restricted epitope was found to persist over the entire observation period, as shown by TCR analysis of cDNA libraries generated from PBMC. The analysis of autologous viral sequences did not reveal any escape mutations within the targeted epitope, and viral load measurement indicated ongoing viral replication. Furthermore, inhibition of viral replication assays indicated that the epitope was properly processed from autologous viral protein. These data demonstrate that persistent exposure to high levels of viral Ag does not necessarily lead to clonal exhaustion and that epitope-specific clonal CTL responses induced within the first weeks of life can persist for years without inducing detectable viral escape variants. The Journal of Immunology, 1999, 162: 4796–4800.

Materials and Methods

Study subject

Virus culture assays at birth indicated that VI-06 had been infected in utero (5, 6). The viral load was measured by the Roche Amplicor assay (Roche Molecular Systems, Branchburg, NJ) with a lower detection limit of 400 viral RNA copies/ml.

Precursor frequency assay

CTL precursor frequencies were determined by limiting dilutions of PBMC, which were either stimulated with peptide-pulsed autologous B lymphoblastoid cell line and irradiated feeder cells or with the anti-CD3-specific mAb 12F6 as described elsewhere (3, 9). The precursor frequency was calculated using the maximum likelihood method (3). The cut off for a positive precursor frequency analysis was set at 50 pCTL/10^6 PBMC over the control value to reflect the lower detection limit that can be achieved with the highest cell input of 16,000 cells per well.

Sequencing of rearranged TCR genes

A SL9-specific CTL clone (VI-06.G5) was generated through limiting dilution cloning from a well (16,000 cells/well) of the 12F6-stimulated precursor frequency assay set up with the sample drawn at the age of 37 mo. The generation and maintenance of T cell clones has previously been described (10). The method for determining TCR sequences has been described in detail and was performed with a few modifications (2). mRNA was isolated, reverse transcribed, and anchored PCR was performed after tailing the cDNA with a 5’ polyG tail (terminal deoxynucleotidyl transferase from Life Technologies, Gaithersburg, MD). The resulting PCR product was cloned into the pAMP-1 vector (ClonAmp, Life Technologies), and DNA preparations from single colonies were sequenced. Fourteen (α-chain) and 11 (β-chain) plasmid preparations were sequenced and were all of the same sequence demonstrating the monoclonality of VI-06.G5. These sequence data are available from GenBank under accession numbers AF033824 and AF033825.

Determination of TCR transcript frequencies

TCR transcripts of the VI-06.G5 clone were detected in TCR libraries generated from PBMC samples drawn at different time points after birth. From these PBMC samples, RNA was isolated, reverse transcribed, and anchored using the Clontech Marathon anchor ligation kit (Clontech, Palo Alto, CA). The libraries were generated by amplification with a 3’ end primer located in the constant region of the TCR β-chain (primer 3cb386, CAU CAU CAU CAU GCT CTA CCC CAG GCC TCG GC) and either 1) an anchor-specific 5’ end primer or 2) a primer that amplified the clone-specific Vβ transcripts only (VI-06.G5*-specific Vβ, primer BV645-5, CUA CUA CUA TTC CAG AAT GAA GCT CAA CTA GA). The

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Received for publication December 2, 1998. Accepted for publication January 19, 1999.

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This work was supported by grants from the National Institutes of Health (AI39966 and U19AI38584) and the Schweizerische Stiftung für Medizinisch Biologische Forschung. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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PCR products were then cloned into the pAMpl vector using the CloneAmp system (Life Technologies). These libraries were probed either with a biotin-labeled oligonucleotide that binds to the clonal-specific Vβ-chain or with an oligonucleotide corresponding to the CDR3 region of the VI-06 G5 TCR transcript. This procedure yields 1) frequencies of clone-specific Vβ-chain transcripts among all TCR β-chain transcripts and 2) when using the CDR3-probe, frequencies of the clonal-specific CDR3 sequences among all specifically Vβ amplified transcripts. The final clone-specific frequency among all TCR transcripts is then obtained by multiplying the two frequencies.

**Probing with Vβ- and CDR3-specific oligonucleotide**

At least 270 colonies were picked from each transformation described above and plated on a 155-mm plate (No. 3025; Falcon, Becton Dickinson, Lincoln Park, NJ). Colonies were transferred to nylon membranes (NEF-978Y; NEN Life Science Products, Boston, MA), denatured, and hybridized with either the Vβ- or the CDR3-specific biotin-labeled oligo. After stringent washing, bound biotin-linked oligonucleotides were detected with the Southern light chemiluminescent detection system for biotin-labeled probes according to the manufacturer’s instructions (Tropix, Bedford, MA). Controls for the entire procedure were run in parallel and included positive and negative controls for RT-PCR and the hybridization step. Positive colonies in the chemiluminescence reaction were identified and their inserts sequenced using previously published primers cβ36 and SP1 (3).

**Sequencing of viral DNA**

Proviral DNA was extracted from frozen PBMC pellets for the 12- and 36-mo time point and from a first-round virus isolate for month 24 and used in serial dilutions in a nested PCR reaction. The lowest detectable target sequence copy number in the end point-diluted sample was used for PCR amplification. Outer 5’ long terminal repeat (nucleotides 768–789) and 3’ gag (nucleotides 1022–1047) primers and inner 5’ gag (nucleotides 1–23) and 3’ gag (nucleotides 622–642) primers were used. The same primers were used to sequence the resulting PCR product in both directions. Sequence data are available from GenBank under accession numbers AF060031 through AF060073.

**Inhibition of viral replication**

Inhibition of HIV-1 laboratory-adapted virus strains or primary isolate from subject VI-06 by coculture with SL9-specific CTL clone was tested in a replication inhibition assay as previously described (11). In brief, primary CD4+ cells were generated from an HLA A27, HIV-seronegative donor using an anti-CD3- and anti-CD8-bispecific mAb and infected with HIV-1 (multiplicity of infection, 10-3) tissue culture ID 50/cell). The SL9-specific CTL clone 115.D4 was added at an E:T ratio of 0.5:1. At indicated time points, 1 ml culture supernatant was removed for HIV-1 p24 Ag quantitative ELISA measurement (DuPont, Boston, MA).

**Results**

**Epitope-specific CTL precursor frequencies**

To investigate the fate of epitope-specific CTL responses in the presence of high viral load and to correlate these responses to viral sequence variation, we determined CTL activities against different HIV-1-derived, optimal HLA class I-restricted epitopes in blood samples from subject VI-06. In early studies, this subject was shown to have HIV-1 Gag-specific CTL responses in cord blood; however, the fine specificity and the persistence of this response has not been determined. The viral load, CD4 cell numbers, and different drug regimens prescribed to subject VI-06 are shown in Fig. 1. Despite the early initiation of anti-viral therapy, the subject had a viral burden ranging from 10,000 to 141,000 RNA copies/ml from the earliest time point analyzed, and this high level viremia persisted until the institution of AZT/3TC/and nelfinavir at month 47, at which time the viral load became persistently undetectable.

To determine the frequencies of epitope-specific CTL, limiting dilution analyses were performed using both an anti-CD3 mAb as a stimulus for T cell proliferation and Ag-specific T cell stimulation (3, 9). Because CTL responses measured against entire HIV-1 proteins may represent multiple specificities, the fine specificity of these responses were determined to correlate CTL activity and persistence of clonal CTL responses with sequence variation and control of viral replication. To this end, optimal HLA class I-restricted CTL epitopes previously demonstrated to be involved in anti-HIV-1 CTL responses in adults and to be presented by the HLA alleles expressed by subject VI-06 were selected for these studies (Table I and Ref. 12). Testing samples from time points between 4 and 37 mo of age, persistent responses against the HLA-A*0201-restricted SL9 epitope from HIV-1 Gag-p17 (SLYNT, aa 77–85) could be detected independently of the mode of in vitro stimulation. The frequencies of SL9-specific CTL exceeded 200/106 PBMC at month 4 and reached precursor frequencies of 3300/106 PBMC at month 33 (~1 SL9-specific CTL in 150 CD8+ T cells). In addition to the SL9 response, other CTL activities were detectable against epitopes in Env, Gag, and reverse transcriptase (Fig. 2). The other HLA-A*0201-restricted response against the reverse transcriptase-derived epitope VL9 was consistently detectable and almost always weaker than the SL9-specific response, consistent with what has been observed in HIV-1-infected adults (13, 14). Another, weaker response against an epitope

![FIGURE 1. Viral load, CD4/CD8 counts, and drug regimen in subject VI-06. Subject VI-06 developed thrombocytopenia at 6 wk but no other clinical manifestation of HIV-1 infection since and was additionally treated with high titer HIV Ig from month 45 through month 52. Viral load was undetectable (<400 copies/ml) after month 50.](http://www.jimmunol.org/)

Table I. HLA class I-restricted epitopes testeda

<table>
<thead>
<tr>
<th>HLA Restriction</th>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>HLA-A*0201</td>
<td>Gag-SL9</td>
<td>SLYNTVATL</td>
</tr>
<tr>
<td>RT-VL9</td>
<td>(p17, aa 77–85)</td>
<td></td>
</tr>
<tr>
<td>RT-I9</td>
<td>(RT, aa 346–354)</td>
<td>V1YQMDDL</td>
</tr>
<tr>
<td>RT-I9</td>
<td>(RT, aa 476–484)</td>
<td>ILKEPVHGV</td>
</tr>
<tr>
<td>HLA-B7</td>
<td>Env-RH10</td>
<td>RPNNTRKSI</td>
</tr>
<tr>
<td>HLA-Cw4</td>
<td>Env-SP9</td>
<td>SFNCGGEFF</td>
</tr>
</tbody>
</table>

| a HLA-type of subject VI-06: HLA-A*0201, A23, B7, B51, and Cw4. |
in the V3 loop region of HIV-1 gp120 (RI10, gp120 a.a. 302–312) was intermittently detectable (Fig. 2). These data indicate that subject VI-06 was able to generate CTL responses against HIV-1 early in life with specificities and magnitudes comparable to those observed in adults (13).

Persistence of epitope-specific clonal CTL responses

The dominant CTL response against the HLA-A*0201-restricted SL9 epitope was further analyzed by generating a SL9-specific CTL clone from subject VI-06, sequencing its rearranged TCR genes, and determining the TCR transcript frequencies in PBMC samples from different time points. The CTL clone (designated VI-06.G5) was isolated by limiting dilution cloning from a month 37 sample, a time point when VI-06 had a plasma viral RNA level of 8900 copies/ml. Sequencing of the TCR genes revealed AV4S1-Ja37 usage for the α-chain and BV6S4A1-Jb1.1 for the β-chain, respectively (Table II). Having determined the TCR sequence, serial cDNA libraries generated from PBMC obtained at different time points were tested for the frequency of the VI-06.G5 TCR β-chain transcript (Table III). Using in a first step a specific probe for the variable segment of the β-chain (BV6S4A1), the frequency of BV6S4A1 transcripts among all TCR transcripts (CD4 and CD8 T cells) was determined. In a second step, using a probe that binds specifically the CDR3 region of VI-06.G5 TCR transcripts, the frequency of clone-specific CDR3 transcript sequences among all BV6S4A1 transcripts was determined. The final frequency of clone VI-06.G5-specific TCR β-chain transcript among all TCR transcripts in four samples obtained between 3 and 60 mo ranged from 1/1228 TCR transcripts specific for VI-06.G5 to 1/285 transcripts (corresponding to 814 and 3508 VI-06.G5 cells per 10^6 total T cells, respectively). These data are similar to the frequencies obtained by functional CTLp assays and indicate that clone VI-06.G5 persisted for at least 57 mo in the PBMC of subject VI-06.

Autologous HIV-1 Gag sequences and inhibition of replication of autologous virus

Clonal exhaustion of specific CTL in mice has been described to result from strong expansion of specific CTL in the presence of

<table>
<thead>
<tr>
<th>Table II. TCR sequence of SL-9-specific clone VI-06.G5</th>
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<tbody>
<tr>
<td>V Region</td>
</tr>
<tr>
<td>α-chain</td>
</tr>
<tr>
<td>β-chain</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A</th>
<th>S</th>
<th>S</th>
<th>L</th>
<th>L</th>
<th>G</th>
<th>G</th>
<th>G</th>
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<th>T</th>
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<tbody>
<tr>
<td>.</td>
<td>GCC</td>
<td>AGC</td>
<td>AGC</td>
<td>TTA</td>
<td>TTA</td>
<td>GGG</td>
<td>GGA</td>
<td>GCA</td>
<td>GGG</td>
<td>ACA</td>
<td>GTC</td>
<td>AAC</td>
<td>ACT</td>
<td>GAA</td>
<td>GCT</td>
</tr>
</tbody>
</table>
continuously high viral load (15). This would suggest that virus-specific CTL should persist only under conditions of low viral load. Because genetic variation in the SL9 coding region of HIV-1 Gag-p17 could lead to nonrecognition by CTL and therefore could theoretically prevent clonal exhaustion, autologous virus from subject VI-06 was sequenced and the processing of the CTL epitope from autologous viral Gag protein was analyzed. The sequencing of autologous proviral DNA from VI-06 PBMC, and an autologous viral isolate revealed the exclusive presence of the SL9 index sequence (SLYNTVATL) at all three time points analyzed (Table IV). Because clone VI-06.G5 was screened based on responses to the index sequence, and thus recognizes the wild-type epitope (data not shown), these data indicate that the clone may have been continuously exposed to viral Ag that did not escape recognition. However, viruses may escape CTL recognition not only by changes in the presented epitope but also by changes in the epitope flanking sequences that abrogate the proper processing of SL9. To demonstrate that the SL9 epitope was indeed processed from the autologous Gag-p17 protein and presented on HLA-A*0201 in infected cells, we infected HLA-A*0201-positive CD4 cells from a non-HIV-1-infected individual with a VI-06 viral isolate obtained at month 24 and tested whether SL9-specific clones could inhibit viral replication. Fig. 3 shows the complete suppression of virus replication by a SL9-specific clone in a 10-day coculture system previously shown to mediate its effect through epitope-specific lysis of infected cells (11). This indicates that the infected cells very efficiently processed and presented the SL9 epitope on HLA-A*0201.

Discussion

The data presented here demonstrate the persistence of a CTL clone in PBMC of an in utero HIU-1-infected infant for almost 5 years despite a continuous high level of plasma viremia. The autologous viral Gag sequences and replication inhibition assays demonstrate that the viral Ag has neither escaped CTL recognition nor Ag processing and suggest that the SL9-specific CTL clone has constantly been exposed to immunogenic peptide. At least under the conditions encountered in the studied subject, these data provide strong evidence against clonal exhaustion by exposure to persisting high Ag burden.

Although it is not known to what extent the analyzed clone VI-06.G5 represents the population of SL9-specific CTL in subject VI-06, it was continuously detectable at high frequencies. Because high frequencies of SL9-specific CTL correlate with control of viremia, at least when measured by tetramer staining of epitope-specific CTL (16), the lack of control of viremia in VI-06, suggests a state of “unresponsiveness” of the VI-06.G5 population in vivo. The limited amount of blood samples did not allow for testing freshly isolated, unstimulated PBMC at high E:T ratios to analyze the ex vivo activity of these cells (10). Our ability to detect SL9-specific CTL activity in vitro may be a result of providing help through the addition of IL-2 and feeder cells to the cell cultures. A lack of Th cell activity in vivo may explain the high level viremia despite the abundance of this clone (17). Subject VI-06 may never have developed such Th cell responses or may have lost them over time. As a result, the in vivo CTL population, including the SL9-specific CTL, could be in a state of unresponsiveness, resulting in a reduced effectiveness of these CTL in vivo. However, although it was not able to clear the virus, the CTL response induced after birth could, at least partly, be responsible for the viral steady state observed before the institution of aggressive drug regimen at month 47.

It could be argued that a state of in vivo unresponsiveness may not only have reduced in vivo effectiveness of VI-06.G5 but also protected it from clonal exhaustion. In addition to lack of Th cell activity, this unresponsive state could also be due to different Th1/Th2 balance (18).

Table IV. Subject VI-06 autologous viral sequences in Gag-p17 (77–85)

<table>
<thead>
<tr>
<th>Time Point (mo)</th>
<th>Viral Load</th>
<th>Sequences Analyzed</th>
<th>Epitope (Variants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>30321</td>
<td>13/13</td>
<td>SLYNTVATL</td>
</tr>
<tr>
<td>24</td>
<td>12300</td>
<td>13/13</td>
<td>SLYNTVATL</td>
</tr>
<tr>
<td>36</td>
<td>8786</td>
<td>9/9</td>
<td>SLYNTVATL</td>
</tr>
</tbody>
</table>
Th2 polarization that has been described in neonatally induced immune responses, making the CTL response rather ineffective (18–20). An ineffective CTL response would also be consistent with the homogeneity in autologous virus sequences and the striking lack of sequence variation in the targeted epitope.

Alternative mechanisms of “unresponsiveness” that have been described, such as anergy and split tolerance, may also have reduced the effectiveness of V1-06′ SL9-specific CTL response in vivo (18, 21–23). Furthermore, the distinct clinical outcome of in utero/neonatal infection in humans and mice with CMV, herpes simplex, herpes zoster, and lymphocytic choriomeningitis virus suggest a state of immaturity of the neonate immune system, and a number of studies have demonstrated the induction of different immune responses depending on the age at Ag exposure (i.e., different specificities, tolerance induction, clonal exhaustion) (reviewed in Ref. 24) (15, 19, 20, 25–29). In our case, infection occurred in utero, as cord blood samples were positive for HIV-1. However, infection must have occurred after this subject reached immunocompetence, because HIV-Gag-specific CTL were present in the cord blood. This also rules out central tolerance induction, which is described to occur after around week 7–10 of gestation (5, 30).

In summary, we suggest that although clonal exhaustion may occur after adequate stimulation and differentiation of T cells in animal models in the presence of high Ag concentration (15, 22), it seems that neonatal (CTL) responses that develop in the presence of persistent Ag are not necessarily subject to this fate. Further detailed studies in adults will be required to determine whether this lack of clonal exhaustion is specific for the neonate immune system or whether clonal persistence in the presence of high concentration of immunogenic Ag can occur in individuals infected after their immune system has matured.

Acknowledgments

We thank Kevin Byron and Dena Giokas for their excellent technical support. The IL-2 was a kind gift from Dr. M. Gately and Hoffmann-LaRoche (Nutley, NJ).

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