Virus-Specific CD8\(^+\) Cytotoxic T-Lymphocyte Activity Associated with Control of Viremia in Primary Human Immunodeficiency Virus Type 1 Infection

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Human immunodeficiency virus type 1 (HIV-1) Env-, Gag-, Pol-, Nef-, and Tat-specific cytotoxic T-lymphocyte (CTL) activities were quantitated temporally in five patients with symptomatic primary HIV-1 infection. A dominant CD8\(^+\) -mediated, major histocompatibility complex I-restricted CTL response to the HIV-1 envelope glycoprotein, gp160, was noted in four of the five patients studied. The level of HIV-1-specific CTL activity in the five patients paralleled the efficiency of control of primary viremia. Patients who mounted strong gp160-specific CTL responses showed rapid reduction of acute plasma viremia and antigenemia, while in contrast, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. These results suggest that HIV-1-specific CTL activity is a major component of the host immune response associated with the control of virus replication following primary HIV-1 infection and have important implications for the design of antiviral vaccines.

Human immunodeficiency virus type 1 (HIV-1) infection has reached pandemic proportions (reviewed in reference 27). To design successful prophylactic and therapeutic strategies to combat this lethal infection, an understanding of the mechanisms by which HIV-1 replication is naturally controlled is of vital importance. The immune system has a critical role in containment of virus infections (3, 28, 46). Both humoral and cell-mediated immune responses are known to be mounted to HIV-1, but the contribution of either component to protection is not known. In other virus infections, cell-mediated immunity, in particular the antiviral cytotoxic T-lymphocyte (CTL) response, plays a key role in virus control (4, 26, 34, 38, 47); and there is preliminary evidence to suggest this may also be the case in HIV-1 infection (2, 9, 10; for reviews, see references 30 and 44). Activated HIV-1-specific CTLs have been shown to circulate at remarkably high frequency in chronically infected individuals (16, 33, 42), and CD8\(^+\) lymphocytes have been shown to inhibit HIV-1 replication both in vitro and in a SCID mouse-human peripheral blood mononuclear cell (PBMC) model (19, 29, 40, 45). Other studies show a positive correlation between virus-specific CTL activity and clinical status in chronically-infected individuals (reviewed in references 30 and 44), although these are difficult to interpret because of the immunosuppressive nature of the disease. The protective capacity of antiviral CTLs during HIV-1 infection thus remains poorly understood.

To better grasp the role of the HIV-1-specific CTL response, we measured levels of CTL activity to different HIV-1 proteins from the onset of primary symptomatic HIV-1 infection, when high titers of infectious virus are found in plasma (7, 11, 32). We noted that those patients who controlled their plasma viremia within 3 to 4 weeks after the onset of the acute retroviral syndrome generated significant CD8\(^+\), major histocompatibility complex (MHC)-restricted anti-HIV-1 CTL responses. The predominant response was to gp160, although responses to other HIV-1 proteins, Gag and Tat, were also made. By contrast, those patients who either failed to make anti-HIV-1 CTL responses or mounted low responses did not efficiently control primary viremia and antigenemia.

The five patients in this study presented to hospital emergency rooms and were evaluated prospectively at the University of Alabama, Birmingham. Approval from the human subjects review board and informed consent were obtained. All five patients presented with signs and symptoms of primary HIV-1 infection and no detectable HIV-1 reactive antibodies as determined by enzyme-linked immunosorbent assay or protein immunoblot. However, each subsequently seroconverted with a full spectrum of HIV-1-specific antibodies by day 50. The clinical histories of patients WEAU, BORI, and INME are described by Clark et al. (7), and detailed kinetics of the plasma viremia, p24 antigenemia, and CD4\(^+\) cell counts of patients WEAU, BORI, SUMA, and INME are reported by Clark et al. (7) and Piatak et al. (32). Three of the five patients (WEAU, BORI, and SUMA) controlled plasma viremia rapidly and efficiently. They cleared infectious virus from the plasma by 3 1/2 weeks after the onset of the acute retroviral syndrome and maintained control of infectious viremia and antigenemia for more than a year (Table 1). By contrast, the other two patients (INME and HOBR) controlled primary plasma viremia and antigenemia less efficiently. They both took longer (up to 217 days) to clear infectious virus from the plasma, and neither of them eliminated circulating p24 antigen. Further, in HOBR, infectious viremia remained negative for an interval of less than 120 days (Table 1).

Analysis of HIV-1-specific CTL activity was performed on sequential PBMC specimens from these patients by chromium-51 (\(^{51}\)Cr) release assays. Blood specimens were collected in acid-citrate-dextrose and processed within 3 h of phlebotomy. PBMC were isolated by standard Ficoll-Hypaque density gra-

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TABLE 1. Summary of virologic features of the five patients studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time at which infectious virus was first observed to be cleared from the plasma</th>
<th>Mean level of plasma ICD-p24 antigen (pg/ml) from 6 to 20 mo after onset of acute retroviral syndrome</th>
<th>Interval before infectious virus was first observed to reappear in the plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEAU</td>
<td>23</td>
<td>0</td>
<td>368</td>
</tr>
<tr>
<td>BORI</td>
<td>14</td>
<td>0</td>
<td>542</td>
</tr>
<tr>
<td>SUMA</td>
<td>20</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>INME</td>
<td>81</td>
<td>58.3</td>
<td>ND</td>
</tr>
<tr>
<td>HOBRI</td>
<td>217</td>
<td>855.4</td>
<td>126</td>
</tr>
</tbody>
</table>

* Patients are described in references 7 and 32.

* Time measured as days past onset of the acute retroviral syndrome.

* Infectious virus was measured as described in Clark et al. (7).

* Immune complex-disassociated (ICD-p24) antigen levels were determined as described in Piatak et al. (32). Values shown are the mean levels over all time points tested between 6 and 20 months after the onset of the acute retroviral syndrome.

* Number of days from time at which infectious virus was first observed to be cleared from the plasma until infectious virus was first observed to reappear in the plasma.

* ND indicates that the length of the interval before reappearance of infectious virus in the plasma is unknown, i.e., infectious viremia was not observed to reappear in the plasma at any of the time points tested (up to day 483 for SUMA and day 553 for INME).

The patient's viral load was measured using an autologous and allogeneic B-LCL target cells infected at a multiplicity of infection of 5 to 10 PFU per cell 16 h prior to the assay were 51Cr labeled by incubation of 10^6 cells with 100 μCi of Na25CrO4 (Amersham, Arlington Heights, Ill.) in a 100-μl volume for 1 h at 37°C, washed four times, and resuspended in R10 medium at 10^5 cells per ml. Target cells were then incubated at 10^4 cells per well in 96-well flat-bottomed plates with patient PBMC at effector-to-target (E/T) cell ratios between 100:1 and 1:10, in a total volume of 200 μl at 37°C in 5% CO2. Control wells to which R10 medium only or 1% Nonidet P-40 were added were allowed assessment of spontaneous and normal 51Cr release in each assay. After 5 h, 100 μl of supernatant was harvested from each well and the radioactivity was determined. The percent specific 51Cr release was calculated as 100 × (experimental release – spontaneous release)/ (total release – spontaneous release). All variables were tested in triplicate; each result shown is the mean percent specific 51Cr release. Figure 1 illustrates data from a typical CTL assay.

CTL activity specific for a particular HIV-1 protein was considered present if there was at least 10% greater specific lysis of target cells infected with the recombinant vaccinia virus encoding it than of those infected with the control vaccinia virus expressing β-Gal only. For example, the effector population in the experiment shown in Fig. 1 exhibited both gp160- and Gag-specific CTL activity. Whereas Gag-specific CTL lysis was decreased as the E/T cell ratio was reduced from 80:1 to 10:1, the level of gp160-specific CTL activity was so high that similar levels of gp160-specific lysis were observed at E/T cell ratios of 80:1 and 10:1, illustrating that at the E/T cell ratios used, the assay was nonquantitative at these high CTL activity levels.

HIV-1-specific CTL activity was MHC restricted, as lysis of allogeneic target cells infected with vaccinia virus recombinants expressing HIV-1 proteins was low or undetectable (Fig. 1b).

The HIV-1-specific CTL activity present over time following the onset of the acute retroviral syndrome associated with primary HIV-1 infection was analyzed in all five of the patients for whom data are shown in Table 1, and in two of these, analysis was continued throughout the course of the infection. Figure 2 summarizes the results obtained. Focusing first on the response at early times after the onset of the acute retroviral syndrome, high levels of gp160-specific CTL activity were detected in patients WEAU, BORI, and SUMA from the first time point examined. Patient BORI also exhibited Gag-specific CTL activity at all time points tested, and patient SUMA exhibited Tat-specific CTL activity. CTL activities specific for other HIV-1 proteins were not consistently observed in these patients. In patient INME, gp160-specific CTL activity was also detected at all time points tested, but at a lower level than in patients WEAU, BORI, and SUMA: the mean (± standard error) gp160-specific 51Cr release mediated by INME effectors at all time points tested in the first 100 days after the onset of the acute retroviral syndrome was 22.5 ± 1.4 at an E/T cell ratio of 100:1, whereas the corresponding values for WEAU, BORI, and SUMA were 37.2 ± 1.4, 75.2 ± 2.1, and 42.5 ± 3.1,
respective. No HIV-1-specific CTL activity was detected in early samples from patient HOBR, although this patient did exhibit some CTL activity directed against vaccinia virus antigens. That CTL activity to vaccinia virus was retained by HOBR's PBMC after the in vitro culture period indicated that there was not a generalized immune suppression or inability of PBMC to expand in vitro in this patient, but rather a specific lack of anti-HIV-1 CTL reactivity. Although envelope sequence analysis suggests that the HIV-1 of patient HOBR is not more divergent from laboratory strains of HIV-1 than those of the other four patients studied (39), single amino-acid changes can abolish CTL recognition. We thus cannot rule out that the lack of detection of HIV-1-specific CTL activity in patient HOBR is due to the HIV-1 isolates present in this patient at early times postinfection having sequences sufficiently divergent from the laboratory strain HIV-1 sequences in the vaccinia virus recombinants used for the detection of HIV-1-specific CTL activity for the response not to be cross-reactive. This possibility is being addressed by expression of the envelope genes of early virus from patient HOBR in a vaccinia virus vector to provide target cells expressing autologous virus sequences.

In patients WEAU and BORI, in whom HIV-1-specific CTL activity was followed longitudinally throughout the course of the infection, the initial pattern of CTL activity was maintained for more than 200 days after the onset of the acute retroviral syndrome. Changes in the frequency of HIV-1-specific CTLs may have occurred over this time frame, particularly in patient BORI, for as noted above, the CTL assay technique used here was relatively nonquantitative, especially at high activity levels (see Fig. 1). In both patients, however, from the time points at which infectious virus was first found to reappear in the plasma onwards, levels of HIV-1-specific CTL activity became low or undetectable. There are two possible explanations for the lack of detection of HIV-1-specific CTL activity in these patients at later times. One is an absence of HIV-1-specific CTL activity, and the other is a switch in CTL specificity, possibly driven by the emergence of CTL escape virus variants (31) with mutations in the CTL epitopes recognized at early times postinfection. The latter possibility is currently under investigation.

\[ ^{51} \text{Cr} \] release from autologous target cells expressing HIV-1 gp160 can be mediated by CD16\(^+\), MHC-unrestricted effectors in addition to classical MHC-restricted CD8\(^+\) CTLs (21, 23, 36, 41; for a review, see reference 18). As the predominant HIV-1-specific CTL activity detected in the experiments shown in Fig. 2 was gp160-specific, a number of experiments were performed with acute-phase effectors from patients WEAU, BORI, SUMA, and INME to characterize this activity and determine whether it was mediated by classical CTLs. First, we investigated whether the lysis was MHC restricted. Killing of gp160-expressing allogeneic target cells was low or undetectable (illustrated for patient BORI in Fig. 1; similar data for the other patients not shown), indicating that the majority of the CTL activity detected was MHC restricted. Other experiments demonstrated that restriction was to MHC class I rather than MHC class II, as the gp160-specific lysis mediated by effector populations from all four patients depleted of cells with antibody-dependent cell-mediated cytotoxicity activity could be blocked with an MHC class I-specific monoclonal antibody, but not an HLA-DR-specific monoclonal antibody previously shown to block class II-restricted CTL lysis (data not shown).

Secondly, the phenotype of the effector cells mediating gp160-specific lysis was examined by depleting CD4\(^+\) or CD8\(^+\) cells from the effector population from each patient and testing whether the remaining cells retained gp160-specific CTL activity. To deplete patient PBMC effector populations of CD4\(^+\) or CD8\(^+\) T cells, 5 \( \times \) 10\(^7\) cells were resuspended in a 300-\( \mu \text{l} \) volume of subset-specific monoclonal antibody, either anti-CD4 at 100 \( \mu \text{g} \) of purified immunoglobulin (Olympus Corporation, Lake Success, N.Y.) per ml or anti-CD8 at 12.5 \( \mu \text{g} \) of purified immunoglobulin (Becton Dickinson, San Jose, Calif.) per ml, and incubated for 45 min. The cells were then washed once in R10 medium, resuspended in 500 \( \mu \text{l} \) of freshly reconstituted (as per the manufacturer's instructions) baby rabbit complement (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada), and incubated for 30 min at 37\(^\circ\)C.
FIG. 2. Temporal analysis of HIV-1-specific CTL activity in five patients with symptomatic primary HIV-1 infection. PBMC cryopreserved from patients WEAU, BORI, SUMA, INME, and HOBR at the indicated times (days) after the onset of the acute retroviral syndrome were expanded in vitro by polyclonal stimulation, and then their ability to lyse autologous target cells which were uninfected (■) or infected with vaccinia virus recombinants expressing β-Gal (■) or HIV-1 gp160 (■), Gag-Pol (■), Pol (■), Tat (■), or Nef (■) was tested by ⁵¹Cr release assay as described in the legend to Fig. 1. All variables were tested in triplicate. Each result shown is the mean percent specific ⁵¹Cr release from each target at an E/T cell ratio of 100:1. The variance was always <10%. Where there was no lysis of a target, a bar with a value of −1% specific ⁵¹Cr release is shown here, to distinguish targets which were not lysed from targets which were not tested at a particular time point, for which no bar is shown. HIV-1-specific lysis of allogeneic target cells was also tested in all assays (not shown); it never exceeded 20% and was generally much lower. To ensure reproducibility of data from assay to assay, several PBMC samples from different patients were tested more than once in different assays; consistent results were obtained (not shown).
FIG. 3. Depletion experiment to determine the phenotype of the effector cells mediating gp160-specific CTL activity. In two separate experiments, PBMC cryopreserved from patient WEAU 30 days and patient BORI 21 days after the onset of the acute retroviral syndrome were expanded in vitro by polyclonal stimulation for 10 days. These cells were then employed either undepleted or after depletion of CD8+ or CD4+ T lymphocytes by treatment with an appropriate subset-specific monoclonal antibody and complement as effectors in a 51Cr release assay. In the experiment with patient WEAU PBMC, 86% of CD8+ and 100% of CD4+ cells were removed from the CD8+ and CD4+ cell-depleted effector populations, respectively, and in the experiment with patient BORI PBMC the corresponding values were 94% of CD8+ and 100% of CD4+ cells. Lysis of autologous target cells infected with recombinant vaccinia viruses expressing HIV-1 gp160 or β-Gal only was determined as described in the legend to Fig. 1. The results shown are gp160-specific CTL activities at an E/T cell ratio of 50:1, i.e., mean percent specific 51Cr release from gp160-expressing targets minus mean percent specific 51Cr release from β-Gal-only-expressing targets, and each is expressed as a percentage of the lysis mediated by the undepleted effector population, which was 24.8% in the experiment with patient WEAU PBMC and 45.5% in the experiment with patient BORI PBMC.

after which another 500 μl of complement was added and the cells were incubated for a further 30 min at 37°C. The cells were finally washed three times in R10 medium, and viable PBMC were enumerated by trypan blue staining. The efficiency of depletion of CD4+ or CD8+ T cells from effector PBMC populations treated with appropriate T-cell subset-specific monoclonal antibodies and complement was checked by enumeration of viable CD4+ and CD8+ cells in the depleted and undepleted populations by fluorescence-activated cell sorter analysis after staining with propidium iodide and fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 monoclonal antibody (Dako A/S, Glostrup, Denmark), by standard techniques. Representative data from experiments using PBMC from patients WEAU and BORI are shown in Fig. 3; similar results were obtained with PBMC from patients SUMA and INME (not shown). In the experiment with patient WEAU PBMC, CD4+ cells were depleted from 7.5 to 0% and CD8+ cells were depleted from 83.8% (mean fluorescence intensity, 584) to 11.1% (mean fluorescence intensity, 291); and in the experiment with patient BORI PBMC, CD4+ cells were depleted from 13.6 to 0% and CD8+ cells were depleted from 82.7% (mean fluorescence intensity, 488) to 5.2% (mean fluorescence intensity, 289). CD4+ T-cell depletion was thus 100% efficient in both cases, while CD8+ T-cell depletion was more than 85% efficient, and the cells which remained had a much lower mean level of surface CD8 expression (and thus lower avidity for target cells) than the undepleted population. It was found that this depletion of CD8+ cells from the effector population from each patient significantly reduced or completely ablated gp160-specific lysis, whereas the gp160-specific lytic capacity of CD4-depleted effector cells was not impaired (Fig. 3). This indicated that the effector cells mediating gp160-specific lysis were CD8+ CTL.

Thirdly, it was shown that lysis was not directed against whole gp160 expressed on the target cell surface, as effectors from the four patients exhibiting acute-phase gp160-specific CTL activity lysed target cells infected with vaccinia virus VVTG 3183, which expresses a modified gp160 molecule with a 28-amino-acid deletion of the N-terminal signal sequence which is not displayed on the cell surface (25), or vPE8, which expresses just gp120 (13) with efficiency similar to that of target cells expressing the whole of gp160 (Table 2). Further, effectors from patient BORI were also able to recognize autologous target cells coated with a synthetic peptide spanning gp41 amino acids 585 to 593, ERYLKDOQQL (17) (Table 2). This peptide-specific lysis was MHC restricted: whereas D6 BORI effectors at an E/T cell ratio of 25:1 caused 54.3% specific 51Cr release from autologous target cells coated with peptide 93-1 (74% of the lysis of autologous targets infected with vPE16 in the experiment reported in Table 2), the same effectors mediated only 9.9% specific 51Cr release from allogeneic target cells coated with peptide 93-1 (not shown in Table 2). ERYLKDQQL is thus one of the epitopes recognized by BORI effectors. Whether HIV-1 isolates obtained from patient BORI at early times postinfection actually contain the sequence ERYLKDQQL or a variant of this is not known but is currently under investigation.

In summary, patients with symptomatic primary HIV-1 infection who best control their acute viral infection mount strong HIV-1-specific CTL responses at very early times after the onset of the acute retroviral syndrome. CTLs are generated prior to clearance of the acute infectious viremia. In the patients we studied, the early antiviral CTL response was predominantly directed against gp160 and was mediated by CD8+, MHC class I-restricted CTLs. Our results contrast with previous reports that Gag-specific CTLs make up a major portion of the antiviral CTL response in many patients chronically infected with HIV-1 (reviewed in reference 18). Whether our observation that the predominant response at early times postinfection was gp160 specific is fortuitous or reflects a real
difference in the specificity of primary CTLs remains to be seen. Interestingly, four CTL clones isolated during acute HIV-1 infection, characterization of which was recently reported (37), all demonstrated specificity for Env of HIV-1 and recognized sequences within gp41. Studies of the early immune events associated with the containment of acute infection of rhesus monkeys with simian immunodeficiency virus of macaques (SIVmac) have also shown that virus-specific CTL activity can be detected at very early times postinfection, prior to control of the initial viremia (48). In this model, whereas SIVmac-specific CTL precursors were detected in both blood and lymph nodes by 7 days post-virus inoculation, anti-SIVmac antibodies were not detected in the blood until approximately day 14, and SIVmac neutralizing antibodies reached significant titers only by days 29 to 34, after containment of acute viremia/antigenemia (which occurred at 3 to 4 weeks) (35). Similarly, a study of the timing of neutralizing antibody production with respect to time of control of primary plasma viremia in patients with acute HIV-1 infection showed that decline of plasma viremia to undetectable levels occurred within 4 to 8 weeks, coincident with seroconversion, but that neutralizing antibodies directed against the first autologous isolates taken during the viremic period could not be detected until after the time of disappearance of plasma virus (1). The authors of reference 1 concluded that it is thus unlikely that humoral factors are responsible for the suppression of primary viremia in early HIV infection (although they may have important roles in the subsequent stages of the infection [6]). Neutralizing antibody production has not yet been assessed in the patients we studied here (although we plan to examine this); however, the time of seroconversion has been measured (7, 32), and in all four patients who mounted CTL responses, CTL activity was detectable prior to seroconversion.

The timing of the virus-specific CD8+ CTL response thus makes it a likely candidate for playing a role in containment of primary viremia. Further, all three patients who exhibited high levels of HIV-1-specific CTL activity at early times after onset of the acute retroviral syndrome (Fig. 2) controlled infectious plasma viremia and antigenemia rapidly and efficiently and maintained this control for more than a year (Table 1). In contrast, viremia and antigenemia were less efficiently controlled (Table 1) in the two patients in whom HIV-1-specific CTL activity was low (INME) or undetectable (HOBR) (Fig. 2). Although the number of patients studied here is limited, the association noted provides evidence that HIV-1-specific CTL activity is a key component of the protective immune response to primary HIV-1 infection and suggests that stimulation of HIV-1-specific CD8+ CTL should be among the goals of future vaccine strategies.

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REFERENCES


### Table 2. Recognition of target cells expressing portions of the HIV-1 envelope glycoprotein by effectors from the four patients with gp160-specific CTL activity

<table>
<thead>
<tr>
<th>Effector</th>
<th>Relative efficiency of lysis of autologous targets presenting:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>gp16</td>
</tr>
<tr>
<td>WEAU D34</td>
<td>100</td>
</tr>
<tr>
<td>BORI D6</td>
<td>100</td>
</tr>
<tr>
<td>SUMA D5</td>
<td>100</td>
</tr>
<tr>
<td>INME D24</td>
<td>100</td>
</tr>
</tbody>
</table>

- WEAU, BORI, and SUMA: PBMC cryopreserved from the indicated four patients at the indicated number of days (D) after onset of the acute retroviral syndrome were expanded in vitro and employed at an E/T ratio of 25:1 (WEAU and BORI) or 50:1 (SUMA and INME) in 51Cr release assays as detailed in the text. Similar results were obtained at lower E/T ratios (not shown).
- Each result shown is the mean percent specific 51Cr release from each target expressed as a percentage of the mean percent specific 51Cr release from target cells expressing the entire HIV-1 envelope glycoprotein (100%). Target cells were autologous B-LCL infected with vaccinia virus either VPE16 (expresses the whole of HIV-1 gp160 [12]), VVTG3183 (expresses HIV-1 gp160 with a 28-amino-acid deletion of the N-terminal signal sequence [25]), or VPE8 (expresses HIV-1 gp120 [13]) or incubated during the 5-h assay period with 100 μg of peptide 93-1 (a B14-restricted gp41 epitope, ERYLKDOOQL [17]) or peptide 91-41 (an A2-restricted Gag epitope, GNFLOSRPEDTAPPF [8]) per ml.

- NT: Not tested.


