Changes in Frequency of HIV-1—Specific Cytotoxic T Cell Precursors and Circulating Effectors after Combination Antiretroviral Therapy in Children

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Combination antiretroviral therapy has had a major role in reducing human immunodeficiency virus type 1 (HIV-1) plasma viral loads in HIV-1-infected adults but a variable effect in infants, in whom complete viral suppression appears to be less readily achieved. In adults, after the reduction in plasma viremia, there is a decrease in the numbers of circulating cytotoxic T cell (CTL) effectors and precursors in the majority of patients. This longitudinal study assessed the effect of combination drug therapy on the frequency of HIV-1—specific CTL responses in 8 HIV-1—infected children. Following treatment, the frequency of HIV-1—specific CTL responses initially increased, especially in children with incomplete viral suppression but with increasing CD4+ cell counts. In children with complete viral suppression, the frequency of HIV-1—specific CTL responses decreased, suggesting that viral replication is required to maintain CTL responses in the systemic circulation.

Most adults in the United States who are infected with human immunodeficiency virus type 1 (HIV-1) are now undergoing antiretroviral drug therapy, and combination therapies that include protease inhibitors have been shown to suppress viral replication for ≥3 years in patients adherent to their drug therapies [1, 2]. These combination antiretroviral drug regimens have had a dramatic impact on AIDS-related illness and mortality. For the first time since the beginning of the pandemic, a 60%–70% reduction in death rates has been reported in New York, California, and New Jersey [3].

Regeneration of immune responses after initiation of combination therapy has been reported, including improvements in proliferative responses to recall [4–7]. HIV [8], and cytomegalovirus antigens [9]. Resolution of HIV-associated hypergammaglobulinemia can also occur [10]. In most patients, the absolute CD4+ T cell count increases. Compared with adult patients receiving combination antiretroviral therapy, children have a more significant increase in CD4+ T cell count after initiation of therapy [11–13], and the rapid increase of naïve CD4+ T cells in children has been attributed to the presence of a functional thymic gland [14, 15]. Despite the increase in CD4+ T cell counts, the duration and persistence of viral suppression in a significant portion of children are less sustained, and drug failure is more common than in adults [11, 13, 16–18].

Studies of HIV-1—infected adults have supported an important role for HIV-1—specific cytotoxic T cells (CTLs) in the control of plasma viremia. These include a temporal association of CTLs with containment of viremia in primary infection [19, 20] and an inverse correlation between plasma load of viral RNA and frequency of HIV-1—specific CTLs [21, 22]. Progression to AIDS in untreated individuals is associated with a decline in frequency of HIV-1—specific CTLs [23–25], which exert an antiviral effect through direct lysis of HIV-1—infected target cells and release of soluble cytokines and chemokines [26, 27]. Because CTLs appear to be critical in containment of viral replication, their loss or reduction could lead to serious adverse consequences. We, and others, recently reported the effect, ascertained by new techniques for measuring the frequency of circulating CTLs [28], of potent combination antiretroviral therapy on the HIV-1—specific CTL response in adults treated with antiretroviral drug therapy [22, 29–31]. In the majority of patients who adhere to their drug regimen, there is a decrease in the number of HIV-1—specific CTLs by 6 months after the
start of therapy. Because HIV-1–specific CTLs appear to be critical in the containment of plasma viremia [19, 20], their loss or reduction could have serious consequences if drug therapy is withdrawn. How such drug therapy affects the HIV-1–specific CTL response in infants and children is little understood.

Previous studies have clearly shown that HIV-1–specific CTLs in pediatric patients can be equivalent in frequency, breadth, and specificity to those found in adults [32–34], although detection of HIV-1–specific CTL responses in the first 6 months of life has been rare [33]. This report describes the effect of combination antiretroviral therapy on HIV-1–specific CTL effector (CTLe) and CTL precursor (CTLp) levels in 8 children with vertically acquired HIV-1 infection, as determined in longitudinal analysis of cryopreserved peripheral blood mononuclear cells.

**Materials and Methods**

**Subjects.** All patients were followed up prospectively by the Pediatric Infectious Diseases Service, New York University Medical Center/Bellevue Hospital (New York City). Children were enrolled in pediatric treatment protocols available at that time. Consent was obtained from parents or legal guardians, and the study was approved by the institutional review board. The patients’ age range at initiation of combination antiretroviral therapy was 2.02–12.7 years.

**Sample processing.** Fresh blood was collected in EDTA vacuum containers. PBMC were isolated by Ficoll-Hypaque centrifugation, aliquoted, and cryopreserved in media containing 10% dimethyl sulfoxide.

**Measurement of plasma HIV-1 RNA viral load.** Viral RNA copy numbers in plasma were determined by reverse transcriptase polymerase chain reaction (PCR; Roche, Nutley, NJ) according to the manufacturer’s instructions, with a lower limit of detection at 400 copies/mL. For a few earlier time points in 3 patients, the 50-μL format of the Chiron 2.0 branched DNA assay (Chiron, Emeryville, CA) was used, which has a lower limit of detection of 10,000 copies/mL.

**Recombinant vaccinia viruses.** Recombinant vaccinia expressing HIV-1 antigens IIIBenv-gp160, IIIBgag-p55, IIINef, and IIIBpol were used in CTL assays (Therion, Cambridge, MA). Vaccinia strain NYCBH was used as a control. The recombinant vaccinia viruses (rVV)s used in the ELISPOT assays (Vac WR-HIV-1 gag [IIIB] vP1287, Vac WR-HIV-1 pol [IIIB] vP1288, and Vac WR-HIV-1 env gp120TM [MN] vP1218) were provided by Virogenetics (Troy, NY). Vac WR-eco gpt was used as control.

**Peptides.** Peptides were provided by Dr. S. Kalams, Massachusetts General Hospital/Partners AIDS Research Center, Peptide/Protein Core Facility, Boston, and the library of overlapping 20mer HIV-1SF2 gp41 p24 peptides from the Medical Research Council (MRC) HIV reagents repository (Repository Reference ARP788.1–788.22, MRC, Potters Bar, UK).

**B cell lines.** Epstein-Barr virus–transformed B lymphoblastoid cell lines (BCLs) were established on all patients by standard techniques [35].

**51Cr release assays.** To assay HIV-1–specific CTL activity, aliquots of 1 × 10⁶ autologous BCLs were resuspended in 0.3 mL of RPMI 1640 containing 2.0 mM l-glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 15% fetal calf serum (R-15) and incubated for 1 h at 37°C with either control vaccinia or recombinant vaccinia expressing HIV-1 antigens. Target cells were incubated with antigen (peptide or rVV) or control, labeled with 100 μCi of chromium (Na[51Cr]-O₄; New England Nuclear, Boston), and incubated overnight. Target cells were then washed 3 times and aliquoted at 5 × 10⁴ cells per well into U-bottom 96-well plates containing different numbers of CTLe. Target cells and CTLe were coincubated at 37°C for 4–6 h; 30 μL of supernatant was harvested from each well to corresponding wells of a 96-well LumaPlate (Packard Instruments, Downers Grove, IL). Each well’s radioactivity was counted in a TopCount Microplate Scintillation Counter (Packard Instruments), and the percentage-specific lysis from each well was calculated according to the following formula: percentage-specific lysis = (test release – spontaneous release)/(maximal release – spontaneous release) × 100%.

**LDA.** The method for quantifying HIV-1–specific CTLp frequency has been described elsewhere [36]. Briefly, 2 LDA plates were set up with input PBMC numbers of 0, 250, 500, 1000, 3000, 6000, 12,000, and 16,000 per well (24 replicates) in R-15 medium with 100 IU/mL of interleukin 2 (R-15-100), and 2.5 × 10⁴ gamma-irradiated allogeneic PBMC and 0.1 μg/mL of anti-CD3 (clone 12F6, from Dr. J. T. Wong, Boston) were added to each well. The medium was changed twice a week by removing half of the supernatant and replacing it with an equal volume of fresh R-15–100, and cultures were maintained for 14 days prior to assay. A standard 51Cr release was performed, with the effectors from the LDA plates split 6 ways. Effector cells were assayed against autologous BCLs infected with either vaccinia control or recombinant vaccinia expressing IIIBenv, IIIBgag, IIINef, and IIIBpol. The assays were performed by use of a BIOMEK-2000 robotic machine controlled by programs written with BIOWORKS software (Beckman Instruments, Beckman Coulter, Fullerton, CA). A modified data analysis method was used [37]. Briefly, in split-well analysis, a well was regarded as positive for antigen-specific CTL activity when the percentage-specific lysis of autologous target cells exceeded the corresponding control target cells by 10% [37]. After the wells were classified as either positive or negative for CTL activity, the number of replicates and the number of negative wells were entered into programs written for Microsoft Excel (Seattle, WA) to calculate the CTLp frequencies and their corresponding 95% confidence intervals according to the maximum likelihood method (Dr. S. Kalams, Boston). All results are presented as HIV-1–specific CTLp frequencies with background vaccinia wild-type CTL activity subtracted. The final results are expressed as the number of CTLp per 10⁶ input PBMC.

From our previous studies of HIV-1–infected untreated patients [1, 20, 30, 38, 39], we have established a descriptive range of strength of CTL responses: low, 11–49 cells/10⁶ PBMC; moderate, 50–99 cells/10⁶ PBMC; and high, >100 cells/10⁶ PBMC.

**HLA class I typing.** Patients were typed for HLA class I by sequence-specific primer PCR at the Histocompatibility Labora-
Streptavidin-phycoerythrin conjugate (Leinco, St. Louis) was resuspended in 30°C with RPMI 1640 containing 1% pooled human serum (PHS) with rVVs at a multiplicity of infection of 2. Cells were then washed twice with RPMI containing 5% PHS, coated and resuspended in the same medium to the wells in 100–200 μL of culture medium and incubated overnight at 37°C in 5% CO₂. The cell numbers were 0.5–1.0 × 10⁶ per well in duplicate or triplicate wells. Wells were washed 4 times with PBS containing 0.05% Tween 20, followed by a 2 h incubation with 50 μL of the secondary antibody (1 μg/mL of Biotin-conjugated anti-IFN-γ mab; Mabtech, Stockholm, Sweden). Plates were washed 4 times in PBS with 0.1% Tween 20 (Sigma). Avidin-bound horseradish peroxidase H (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) was added to the wells, followed by a 1 h incubation at room temperature. The plates were washed 4 times in PBS with 0.1% Tween 20 followed by a 5-min incubation in stable diaminobenzidine (Research Genetics, Huntsville, AL) to develop the reaction. Tap water was added to stop the reaction. The spots were counted by use of a stereo microscope (Stemi 2000; Carl Zeiss, New York) under magnifications of 20×–40×. Only spots with a fuzzy border and a brown color were counted (Larsson et al., manuscript submitted). The IFN-γ-producing cells detected in this assay are CD8⁺ T cells (Larsson et al., manuscript submitted).

Results

**HIV-1–specific CTL responses before combination antiretroviral therapy.** Longitudinal CTL measurements of HIV-1–specific CTLs were made on cryopreserved PBMC samples from 8 HIV-1–infected children who had acquired HIV-1 through vertical transmission. Six of the 8 children showed a dominant HIV-1 gag- and/or pol-directed CTL response. In 2 patients (C-02 and C-03), a neonatal PBMC sample (<1 month from birth) was tested for HIV-1–specific CTL activity. No HIV-1–specific CTLs were detected (figures 1 and 2). Patient C-01 was started on a zidovudine regimen at 3 months of age, and 2 CTLp measurements after 1 year showed a nonspecific HIV-1 pol CTL response at moderate to high frequency (50–128 cells/10⁶ PBMC; figure 1). His sibling, C-02, also had HIV-1–specific CTL activity but with a broader response. HIV Env, Gag, Pol, and Nef antigens were all recognized (figure 1). Four other patients, C-03, C-04, C-07, and A-03, also made HIV-1–specific CTL responses before combination therapy was started. The HIV-1–specific CTL responses seen in these children were similar in magnitude and specificities to those observed in adults by use of the same assay methods [36, 38, 39]. The HLA-B8 Nef tetramer–positive population in patient C-03 was >4% of CD8⁺ T cells at 9 months of age (figure 2). This

### Table 1. Major histocompatibility complex class I haplotypes

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NOTE. ND, not determined.
Figure 1. Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T cell precursors (CTLps) following highly active antiretroviral therapy (HAART) in HLA-identical siblings C-01 and C-02. Shown are longitudinal comparisons of plasma HIV-1 viral load in siblings C-01 and C-02, with branched DNA assay measurements in squares, reverse transcriptase polymerase chain reaction measurements in diamonds (A); absolute CD4+ T lymphocyte counts (B); HIV-1 Env-specific (diamonds), Gag-specific (squares), Pol-specific (triangles), and Nef-specific (crosses) CTLps per 10⁶ peripheral blood mononuclear cells (PBMC) (C); and changes in HAART over time (D). AZT, zidovudine (azidothymidine); ddI, dideoxyinosine; Rit, ritonavir; 3TC, lamivudine; d4T, stavudine; Nev, nevirapine; Nel, nelﬁnavir; Saq, saquinavir.
Impact of HAART on HIV CTLs in Children

Figure 2. Longitudinal cytotoxic T cell (CTL) responses in patient C-03 during dual combination antiretroviral therapy. Shown are human immunodeficiency virus type 1 (HIV-1) RNA viral load (A); absolute CD4+ T lymphocyte counts (B) during therapy; CTL effector frequency as determined by staining of CD8+ T cells with HLA-B8 Nef peptide tetrameric complexes (C); and changes in antiretroviral therapy over time (D). AZT, zidovudine (azidothymidine); ddI, dideoxyinosine.

HLA-B8-restricted Nef epitope appears to stimulate a vigorous CTL response, because similar levels of CD8+ PBMC staining with this tetramer have been observed in HIV-1–infected adults who are HLA-B8–positive (G. S. Ogg et al., unpublished observations). Patient C-06 was unusual in having no detectable HIV-1–specific CTL precombination therapy, despite CD4+ T cell counts >1000/mL.

HIV-1–specific CTL responses after combination antiretroviral therapy. The maximum viral load reduction after initiation of highly active antiretroviral therapy (HAART) was 1.13–2.47 log10 copies/mL plasma. The magnitude of HIV-1–specific CTL response after initiation of antiretroviral therapy appears to depend upon the level of viral suppression attained, suggesting a direct relationship between antigenic stimulation and CTL response. Just before starting triple therapy, patient C-01 had an undetectable CD4+ count, with no HIV-1–specific CTLs detected (figure 1). After he started to receive combination therapy, which included a protease inhibitor, his CD4+ T cell count increased, and he had a temporary decrease in plasma viremia. At this time his Pol-specific CTL response returned, and a de novo Gag response was detected. This Gag response was HLA-B*57 restricted, and Gag-specific CTL recognized the peptide KAFSPEVIPMF in p24 (data not shown). However, the drug regimen did not contain his plasma viremia, which increased to >105 copies/mL. His therapy was changed to a combination of saquinavir, ritonavir, and stavudine, which resulted in a rapid decrease in plasma viremia to below detection, at which time his HIV-1–specific CTL became undetectable (figure 1). The rapid decline in HIV-1–specific CTL response was also seen in his sibling, patient C-02. After nevirapine and zidovudine were added to dideoxyinosine, the plasma viral load in patient C-02 decreased to below detection, and her CTL response decreased to low levels. However, when dual drug therapy was instituted, plasma viremia rose, and so did her HIV-1–specific CTL response (figure 1).

Patients A-03 and C-07 (figures 3 and 4) also had a prompt virological response to the addition of a protease inhibitor to their drug regimen, with reduction of plasma viremia to below detection within weeks of changing therapy. Concomitant with the decline in plasma viremia, HIV-1–specific CTL declined in patient C-07, but after 1 year of viral suppression the patient developed a strong Pol- and moderate Nef-specific CTL response. As the viral load was reduced to low levels in patient A-03, the Gag-specific CTL response dropped to below detection.

The relationship between CTL response and viral load after therapy is illustrated in the CTL response of patient C-06 (figure 4). No CTL response was initially detected. When therapy was changed to nelfinavir, lamivudine, and stavudine, there was an initial drop in viral load, but viral rebound occurred, at which time an HIV-1–specific CTL response was detected. When saquinavir and ritonavir were substituted for nelfinavir, both plasma viremia and the CTL response dropped. This relation-
ship between viral replication and CTL response was also observed in patient C-05. The longitudinal CTL analysis started while patient C-05 was receiving triple therapy, and a Pol-specific response at low frequency was detected. After therapy was discontinued because of acute pancreatitis and hepatitis, the plasma viremia rose. The Pol-specific CTL response increased in frequency, and a new Gag-specific response was detected. With resumption of therapy (nelfinavir, nevirapine, and stavudine), there was a reduction in viral load and a decrease in CTL response to lower levels (figure 5). Patient C-04 had low CTL responses, low viral load, and a low CD4+ T cell count (figure 5). Although the CD4+ count increased gradually in response to combination therapy, the CTL response showed little change. In patient C-03, viral load reached its set point between 2 and 3 years of life, while the patient was receiving dual therapy with dideoxynosine and zidovudine. The HLA-B8–restricted Nef tetramer–positive response declined gradually in parallel with the plasma viral load (figure 2).

**Discussion**

The effect of combination antiretroviral drug therapy on HIV-1–specific CTL responses in infants and children is little understood, and this study was undertaken to assess the effect of such treatment in 8 HIV-1–infected children with varying virological responses to treatment. We made longitudinal quantitative HIV-1–specific CTL measurements on cryopreserved PBMC from 8 HIV-1–infected children, using 3 quantitative assays for HIV-1–specific CTL activity: LDA to measure CTLps, MHC tetrameric complexes, and IFN-γ ELISPOT to measure CTLes. We observed variable changes in CTL frequency. Following treatment, the frequency of HIV-1–specific CTLs initially increased, especially in children with incomplete viral suppression but increasing CD4+ T cell counts. In children with complete viral suppression, the HIV-1–specific CTL response decreased below detection, suggesting that active viral replication is required to maintain CTLs in the systemic circulation at detectable levels.

Few studies have quantified the HIV-1–specific CTL response in infants or followed the longitudinal changes in the evolution of HIV-1–specific CTL responses in children. Difficulties in assay methodology and availability of adequate numbers of PBMC have been two major problems. Newer assays have now been developed to quantitatively assess CTL responses [31] (Larsson et al., manuscript submitted), and in this study we utilized LDA, ELISPOT, and MHC tetrameric peptide complexes to quantify HIV-1–specific CTL responses. The LDA measures a heterogeneous population of CTLs (thought to be predominantly memory or precursor CTLs) that have the capacity to expand and grow during a period of in vitro culture. This assay has been extensively used in the past to quantify CTL responses. CTL responses to multiple specificities can be measured in LDA through use of split culture assay [38], and
Figure 4. Cytotoxic T cell effector changes following highly active antiretroviral therapy (HAART) in patients C-06 and C-07. Shown are longitudinal changes in plasma human immunodeficiency virus type 1 (HIV-1) viral load (A); absolute CD4+ T lymphocyte counts (B); HIV-1 Env-specific (diamonds), Gag-specific (squares), Pol-specific (triangles), and Nef-specific (crosses) spot-forming cells (SFCs) per 10⁶ peripheral blood mononuclear cells (PBMC), as determined by enzyme-linked immunospot assay (C); and changes in HAART over time (D). d4T, stavudine; Ind, indinavir; 3TC, lamivudine; AZT, zidovudine (azidothymidine); Nel, nelfinavir; Saq, saquinavir; Rit, ritonavir; ddC, zalcitabine.

Thus we could measure CTL responses to HIV Env, Gag, Pol, and Nef gene products simultaneously from a single sample. This assay, therefore, gives a broad measurement of the overall HIV-1–specific CTL frequency, and only a small number of cells are required at input, which is important for pediatric studies because the availability of cells is often reduced in comparison with adults. However, it is now known that LDAs underestimate the number of CTLs by 1–2 logs, and newer assays using MHC tetrameric peptide complexes or intracellular cytokine stains [22, 28, 31, 42, 43] have shown CTLs at high frequency in several viral systems, including HIV-1 infection. MHC tetrameric peptide complexes provide a highly sen-
Figure 5. Changes in cytotoxic T cell precursors (CTLps) following highly active antiretroviral therapy (HAART) in patients C-04 and C-05. Shown are longitudinal changes in plasma human immunodeficiency virus type 1 (HIV-1) viral load (A); absolute CD4\(^+\) T lymphocyte counts (B); HIV-1 Env-specific (diamonds), Gag-specific (squares), Pol-specific (triangles), and Nef-specific (crosses) CTLps per 10\(^6\) peripheral blood mononuclear cells (PBMC) (C); and changes in HAART over time (D). DDI, dideoxyinosine; AZT, zidovudine (azidothymidine); Ind, indinavir; 3TC, lamivudine; d4T, stavudine; Nel, nelﬁnavir; Nev, nevirapine.

Specific and speciﬁc method for detecting antigen-speciﬁc CTLs but rely on the prior identiﬁcation of the HLA-restricted viral peptide CTL epitope and expression of the relevant HLA heavy chain. In this study, we used one MHC tetrameric peptide complex, HLA-B8 Nef, to stain PBMC in patient C-03, who is positive for the HLA-B8 allele. The frequency of HLA-B8 Nef tetramer-positive PBMC in the peripheral blood was initially very high, with >4% of CD8\(^+\) T cells being tetramer positive. The decline in HLA-B8 Nef responses occurred gradually over a 6-year period. To our knowledge, studies using MHC tetrameric peptide complexes in infants or children have not been previously reported. Here, using one tetrameric complex, we
have shown that the frequency of HIV-1–specific CTL in children can be at the same level as in adult patients and that cryopreserved PBMC samples can be stained for measurements of CTL frequency in longitudinal samples. As further MHC tetrameric peptide complexes become available, they will be a very useful reagent for following the evolution of CTL responses in children.

The decrease in frequency of HIV-1–specific CTLs following initiation of viral-suppressive combination therapy in children is similar to that seen in adults [30] and is most likely due to rapid reduction in antigenic stimulation, although redistribution of CTLs to lymphoid tissues cannot be ruled out. When there is incomplete viral suppression, the changes in CTL frequency are complex. For example, patient C-01 had an increase in CTL frequency that occurred after continued improvement of CD4+ T cell count, in parallel with rising plasma viremia. After initiation of HAART, children show a more rapid and sustained increase of naïve CD4+ T cells, to greater absolute numbers than in adults [11, 13, 44], and this increase in CD4+ T cells may further support HIV-1–specific CTL responses [8]. When plasma viremia in patient C-01 was completely suppressed, his HIV-1–specific CTL response then dropped to below detection. Patient C-06 also had HIV-1–specific CTLs detected only after a change in antiretroviral therapy, with CD4+ T cell count increasing and continued viral replication. The rapid increase in CD4+ T cells may have supported infection and viral replication in newly proliferating CD4+ T cells [45], but the balance between CD4+ cell destruction and proliferation was altered so that the CD4+ count increased. The increase in frequency of the HIV-1–specific CTL response after combination therapy in these children is likely to be associated with viral antigen–driven CTL proliferation supported by help from CD4+ T cells.

The more gradual decay of HLA-B8 Nef–restricted tetramer-staining CTLs in patient C-03 appears to parallel the decrease in plasma viremia. Although long-term persistence of immunodominant CTL responses has been reported [46], no studies have quantified such an immunodominant HIV-1–specific CTL response over many years. In studies that rely on bulk culture measurements to determine the presence or absence of a response instead of quantifying the CTL response, such a decline could have been missed. One potential reason for the decline in frequency of HLA-B8 Nef–specific CTLs is the outgrowth of virus “CTL escape” mutants. Such a mechanism has been seen in patients who have progressed to AIDS after having a stable immunodominant CTL response for many years [46]. Sequencing of the epitopic region in viral quasispecies over the course of the decline could help determine the presence or absence of escape mutations.

In summary, the effect of antiretroviral therapy on HIV-1–specific CTL responses in children is complex. Following treatment, the frequency of HIV-1–specific CTLs initially increased, especially in children with incomplete viral suppression but with increasing CD4+ T cell counts. In children with complete viral suppression, the frequency of HIV-1–specific CTL response decreased, suggesting that viral replication is required to maintain CTLs in the systemic circulation. Vaccination to boost HIV-1–specific CTL responses could be considered for children receiving suppressive combination antiretroviral therapy, to broaden and increase CTL responses.

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References


