

# Decay Kinetics of Human Immunodeficiency Virus-Specific Effector Cytotoxic T Lymphocytes after Combination Antiretroviral Therapy

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Received 12 June 1998/Accepted 1 October 1998

**Little is known of the changes in human immunodeficiency virus type 1 (HIV-1)-specific effector cytotoxic T lymphocytes (CTL) after potent antiretroviral therapy. Using HLA/peptide tetrameric complexes, we show that after starting treatment, there are early rapid fluctuations in the HIV-1-specific CTL response which last 1 to 2 weeks. These fluctuations are followed by an exponential decay (median half-life, 45 days) of HIV-1-specific CTL which continues while viremia remains undetectable. These data have implications for the immunological control of drug-resistant virus.**

Combination antiretroviral therapy induces significant changes in the total CD8<sup>+</sup> T-cell count (3, 4, 11) which are accompanied by a decrease in the expression of markers associated with cell activation (HLA-DR and CD38) (3). In the absence of detectable viremia, the frequency of HIV-specific CD8<sup>+</sup> T cells is significantly lower at 6 months than at pretrial levels (16), but it is not known whether this loss occurs in the first few days of treatment or over a longer time course. It is also unclear whether the loss is preceded by a transient increase, as observed in some studies of total CD8<sup>+</sup> cell changes (3). Through frequent sample time points, we used HLA-peptide tetrameric complexes to characterize the detailed kinetics of the human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte (CTL) response after potent antiretroviral therapy in eight treated individuals.

HLA-A\*0201 and HLA-B\*3501 tetramers were synthesized as previously described (2) with the immunodominant peptides SLYNTVATL (HIV type 1 [HIV-1] Gag 77-85) (17) and ILK EPVHGV (HIV-1 Pol 476-84) (21) for A\*0201 and DPNPQE VVL (HIV-1 Env 77-85) (19) for B\*3501. HLA heavy chain was expressed in *Escherichia coli* with an engineered C-terminal signal sequence containing a biotinylation site for the enzyme BirA. The 3' primer used for HLA B\*3501 was GGAGT GGGAC TCTACCCTC GGTCCCTAGG GACGTAGTA TA AGACCTA CGTGCTTT TACCACACC TTAGTAGCA A TTCGAATAGTGT. After refolding of heavy chain,  $\beta$ 2-microglobulin, and peptide, the complex was biotinylated by BirA (Avidity) in the presence of ATP-Mg<sup>2+</sup> (Sigma). Following purification by gel filtration and ion-exchange chromatography, tetramer formation was induced by the addition of streptavidin. Use of fluorescently labeled streptavidin (Extravidin-PE, Sigma) allowed the tetramer to be used for staining of antigen-specific CD8<sup>+</sup> T cells.

Tetramer binding is known to correlate well with functional activity including uncultured peptide-specific cytotoxicity (16) and

gamma interferon production (14). Weaker positive correlations exist between tetramer binding and limiting-dilution analyses (precursor frequency analyses) which quantify those CTL able to survive and divide during a period of expansion in vitro. Limiting-dilution analyses generate frequencies of antigen-specific CTL 1 to 2 logs less than those obtained by tetramer binding (14), and so the overwhelming majority of circulating CTL measured by the tetramers are believed to be effector CTL (CTLe) that have more limited proliferative potential. This is consistent with previous studies showing a similar discrepancy between estimates of circulating CTL activity and limiting-dilution analyses (8, 13). The lower limit of detection of antigen-specific CTL using the HLA tetramers is 0.02% of CD8<sup>+</sup> cells, based on the staining of HIV-negative peripheral-blood mononuclear cells (16). The staining is highly specific, such that CTL clones and lines directed to different epitope peptides bound to the same HLA molecule do not stain (5). Tetramer staining was performed on cryopreserved samples that had been frozen in a controlled-rate facility to maximize cell viability (routinely greater than 95%). Staining of fresh and cryopreserved specimens revealed no difference in the fluorescence intensity or frequency of antigen-specific CTL.

Two-thirds of A\*0201-positive individuals recognize the p17<sup>Gag</sup> 77-85 epitope (SLYNTVATL), and most of the remainder respond to the Pol 476-84 epitope (ILKEPVHGV) (9). Therefore, if both responses are measured concurrently, virtually all individuals will have quantifiable responses (9, 16). Furthermore, levels of CTLe to these epitopes have previously been shown to be potentially important, as the frequency of CTLe inversely correlates with the plasma viral RNA load, consistent with a role in the control of plasma viremia (16).

Eight patients with HLA-A\*0201 or B\*3501 were treated with zidovudine (600 mg daily), lamivudine (300 mg daily), and a protease inhibitor (ritonavir [1,200 mg daily], nelfinavir [2,250 mg daily], or indinavir [2,400 mg daily]). All patients showed a fall in plasma viral RNA load to below the limit of detection throughout the study. Three patients were treated within 120 days of infection, and five patients were treated during the chronic asymptomatic phase. Prior to initiation of

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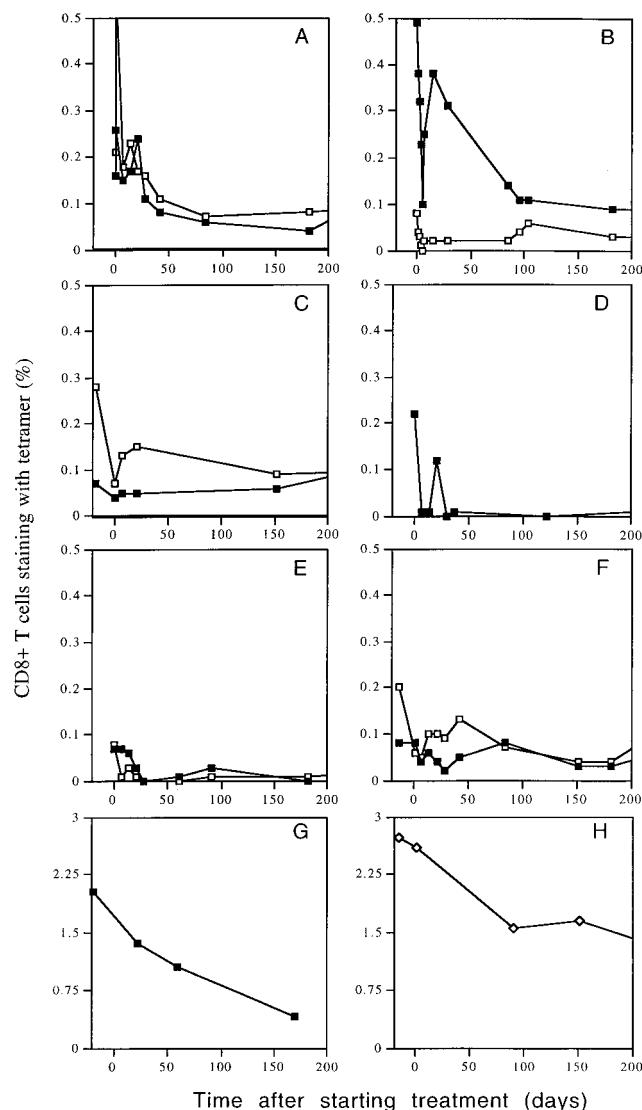


FIG. 1. Changes in HIV-specific CTL frequencies in eight individuals (A to H). The percentages of CD8<sup>+</sup> T cells staining with A2Gag (■), A2Pol (□), and B35Env (◇) are represented on the y axes and the x axes show the number of days after starting treatment. Patients G and H had high frequencies of HIV-specific CTL prior to treatment and therefore have larger-scaled axes than patients A to F. Note that for patients C and F, the first on-therapy time point measured was 1 day after treatment started.

therapy, the CD4 counts ranged from 219 to 564 cells/ $\mu$ l (mean, 357 cells/ $\mu$ l), the CD8 counts ranged from 303 to 2,275 cells/ $\mu$ l (mean, 945 cells/ $\mu$ l), and the plasma RNA viral loads (bDNA; lower limit of detection, 500 copies/ml) ranged from 1,420 to 197,300 copies/ml (mean, 46,327 copies/ml). Patients were HLA typed by using allele-specific PCR.

HIV-specific CTL frequencies were observed in eight individuals after starting treatment with combination therapy. Seven of these individuals were known to be HLA A\*0201 positive, and one was B\*3501 positive. Of the seven HLA A\*0201-positive individuals, all 7 had identifiable A2Gag-specific CTL and six had A2Pol-specific CTL prior to starting therapy. Patients B, D, and E were all treated within 120 days of the onset of symptoms, and patients A, C, and F to H were chronically infected individuals. Figure 1 (A to H) documents the changes, from all patients, in the percentage of CD8<sup>+</sup> T

cells staining with each tetramer for 6 months after initiating treatment. Prior to treatment, the percentage of CD8<sup>+</sup> T cells staining with the tetramer ranged from 0.07 to 2.73% (mean, 0.501%; median, 0.2%), whereas after 6 months of treatment the percentages ranged from 0 to 1.64% (mean, 0.17%; median, 0.04%). Two members of the study cohort (patients G and H) had levels of CTL quantified on more than one occasion prior to treatment, confirming that in the preceding days or weeks, the CTL response was relatively stable in the absence of therapy. We have also observed longitudinal CTL responses in many untreated chronically infected individuals who were not included in the current study and found that large fluctuations do not typically occur in the HIV-specific CTL response over 6 months or more (5a, 10a). This is consistent with previous studies showing that levels of circulating antigen-specific CTL can be maintained for prolonged periods in the absence of treatment (13, 22).

Through frequent sampling in six HLA-A\*0201-positive individuals (Fig. 1A to F), we were able to examine the detailed changes of the HIV-specific CTL in the first 1 to 2 weeks after starting treatment. Large fluctuations in the A2Gag- and A2Pol-specific CTL were observed during the first 2 weeks of therapy. In the majority of individuals, the fluctuations were composed of an initial fall in the first 5 to 7 days, followed by a rebound increase to levels sometimes above those obtained prior to treatment. We reasoned that several factors may have contributed to the early rapid fluctuations in the level of HIV-specific CTL observed after starting treatment, including effects secondary to changes in the CD4<sup>+</sup> T-cell population or redistribution between blood and tissues.

The early rapid fluctuations were then followed by a slower decline phase. Four individuals (patients A, B, G, and H) had pretreatment HIV-specific CTL levels sufficiently high to allow monitoring of the decay of the CTL for several months. Figure 2 documents the decay of HIV-specific CTL in these individuals, which followed an exponential pattern with a median half-life of 45 days (mean, 80 days; range, 40 to 200 days). One individual (patient H) had very high levels of B35Env-specific CTL (2.7% of CD8<sup>+</sup> T cells) prior to starting treatment. We were therefore able to observe the CTL decline in this individual to 20 months on therapy and confirmed that the decay half-life continued to remain constant (Fig. 3A to C). Given the prolonged decay time course, the CTL fall is un-

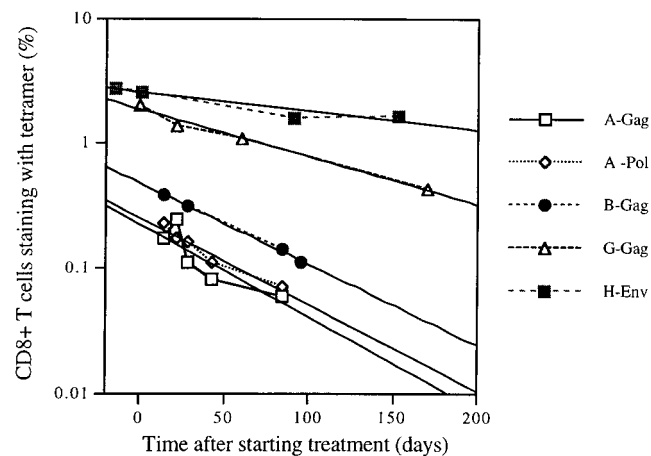


FIG. 2. Late exponential decay of HIV-specific CTL responses in four individuals. The patients and epitopes are identified on the right. Two responses were monitored in patient A, while one response was monitored in patients B, G, and H. The median half-life of the decay was 45 days.

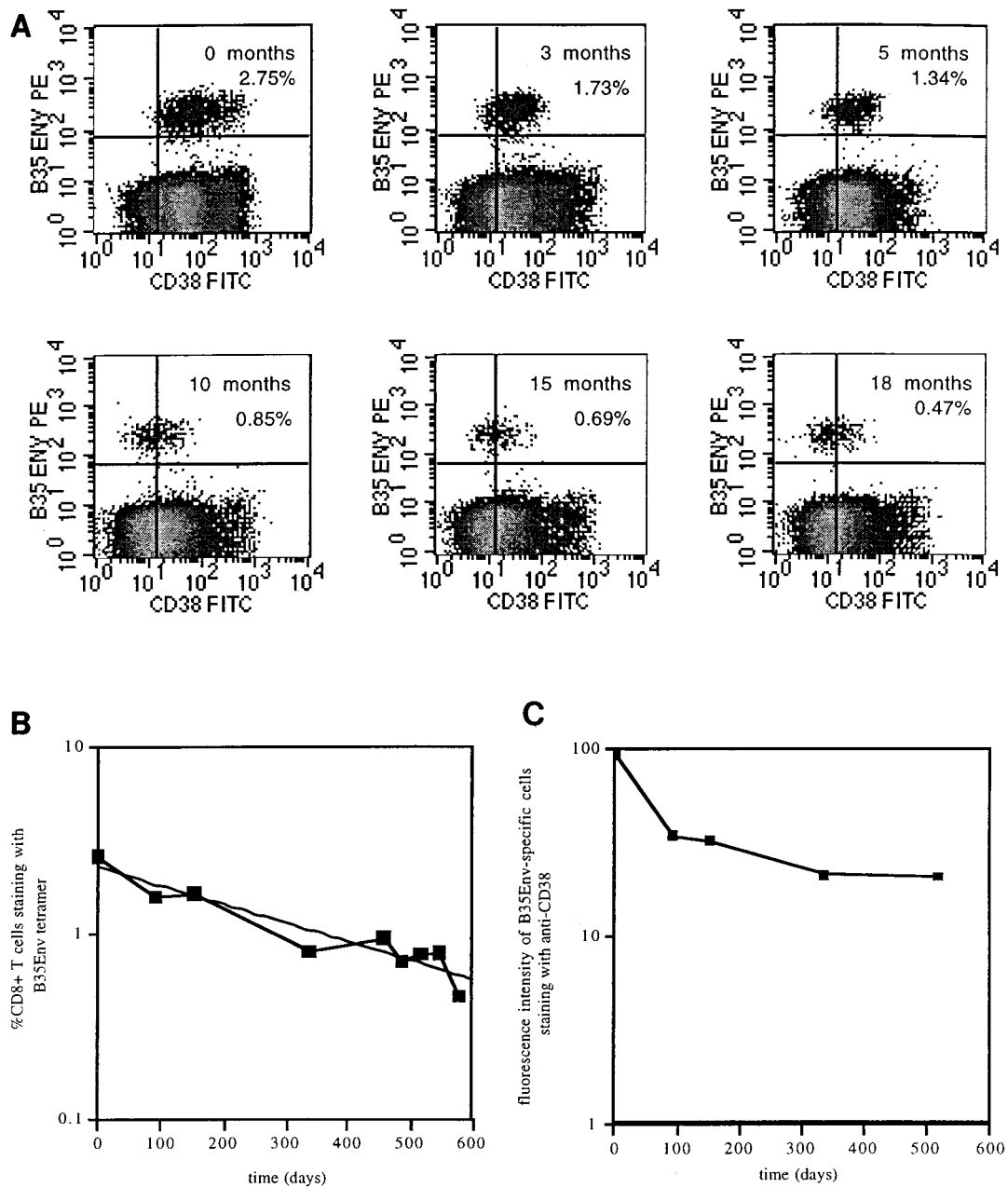


FIG. 3. (A) Serial flow cytometry profiles of one individual after starting treatment with potent antiretrovirals. The CD8<sup>+</sup> T-cell population is illustrated with CD38 expression along the x axes and B35Env tetramer staining along the y axes. The time after treatment and percentage of CD8<sup>+</sup> T cells staining with the tetramer are documented in the top right-hand corner of each graph. The percentage of CD8<sup>+</sup> T cells staining with the tetramer and the intensity with which the tetramer-positive cells stain with anti-CD38 antibodies declined after treatment started. (B) Changes in the percentage of CD8<sup>+</sup> T cells staining with the B35Env tetramer to 600 days after treatment started. The decay half-life remained constant throughout the course of the study. (C) Decay in the staining of tetramer-positive cells with anti-CD38 antibodies. In the absence of detectable viremia, staining with anti-CD38 antibodies declined in intensity.

likely to be explained by redistribution from peripheral blood but instead is more consistent with a real loss of such activated effector CTL. The removal of antigenic drive secondary to the fall in viral replication seems the likely cause for the loss of HIV-specific CTLs, but it is also possible that the drugs might interfere with the presentation of antigen per se or that they are toxic to the CD8<sup>+</sup> T-cell population. However, the increases in the total CD8<sup>+</sup> T-cell number observed in some studies (4, 11) argue that such effects are minimal. All eight individuals had suppression of viremia to below the limit of detection for the duration of the study. It is important to

undertake similar analyses of treated individuals who have had incomplete suppression of viral replication in order to further delineate the causes of the loss of HIV-specific CTLs. Loss of HIV-specific CTLs occurred in all individuals, regardless of the stage of infection. However, none of the patients were treated prior to seroconversion, which has been shown to be important for the protection of HIV-specific CD4<sup>+</sup> T-cell responses (18).

In order to address the question of whether there was continued cell activation during the decay phase, we analyzed the cell surface phenotype of the tetramer-positive cells from all

individuals. CD38 is a cell surface glycoprotein with multiple proposed functions, including a role in adhesion, signalling, and NAD hydrolase activity (20). Intense staining with anti-CD38 antibodies is also known to be a marker of T-cell activation when there is a transient increase in cell surface CD38 (20). The proportion of CD8<sup>+</sup> T cells expressing CD38 increases progressively during the course of infection with HIV (7), and the intensity with which CD8<sup>+</sup> T cells stain with CD38 correlates with plasma viremia (12). Expression of CD38 by the total CD8<sup>+</sup> T-cell population declines during antiretroviral treatment, but it remains unclear whether the same is true of HIV-specific CTL (3). During the HIV-specific CTL decay phase observed after starting treatment, tetramer-positive CTL from all individuals decreased their expression of cell surface CD38 consistent with the loss of their antigenic stimulus and minimal ongoing cell activation. Figure 3A and C shows the decay of cell surface CD38 expression in one individual (patient H). Such a decrease in CD38 expression argues that the exponential loss of HIV-specific CTL reflects the true half-life of these effector CTL in vivo, although it is possible that the net decay phase is apparently slowed by the ongoing proliferation of a small number of HIV-specific clones. Whether such prolonged survival is dependent on antigen persistence (1) is unknown, but it is important to compare the CTL decay rate in individuals with late emergence of drug-resistant virus. Therefore, we conclude that the half-life of HIV-specific effector CTL is less than or equal to 45 days in vivo.

In all treated individuals, levels of HIV-specific CTL fell while viral RNA remained undetectable. This may partly explain why virus can rapidly rise when drug-resistant strains emerge (15). The basic reproductive ratio is defined as the number of secondarily infected cells generated by one infected cell placed into an environment of susceptible cells. This is partially dependent on the host immune system, with an effective response helping to maintain potentially resistant virus in a state where the basic reproductive ratio is less than 1. The eventual loss of circulating HIV-specific CTL activity may weaken such control, leading to the development of resistance. Significant infection of susceptible target cells by drug-resistant virus may occur before memory CTL can proliferate sufficiently to contribute effectively to the overall circulating CTL activity (6). However, it is necessary to study the effects of antiretroviral therapy on the HIV-specific memory CTL response to determine whether a similar loss occurs. Such a loss of memory CTL might be expected to further delay the response to potential drug-resistant virus. An implication of this study is that boosting of HIV-specific CTL by posttreatment vaccination or immunotherapy with cytokines may be an important adjunct to antiretroviral therapy (10). Such strategies could provide a means to maintain effective immunological control of potentially resistant virus.

The assistance of the nursing staff at the Rockefeller Hospital, and that of Jenny Jin and Jia Song in the processing of peripheral blood mononuclear cell samples, is gratefully acknowledged.

This work was supported by NIH U01A141534 and General Clinical Research Center grant MO1-RR00102. G.S.O., S.L.R.-J., and A.J.M. are funded by the MRC UK. S.B. and M.A.N. are supported by the Wellcome Trust.

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