A Semiquantitative Assay for CD8\(^+\) T-Cell-Mediated Suppression of Human Immunodeficiency Virus Type 1 Infection

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A reproducible, semiquantitative assay was developed to measure the level of the anti-human immunodeficiency virus type 1 (anti-HIV-1) suppressive activity of CD8\(^+\) T cells. The assay had a wide dynamic range and could be applied to a relatively small number of fresh and cryopreserved peripheral blood mononuclear cells and purified CD8\(^+\) T cells. The suppressive activity was not due to cytolytic activity and was not major histocompatibility complex class I restricted. Suppression of HIV-1 infection by CD8\(^+\) T cells was consistently demonstrable with both endogenously infected autologous CD4\(^+\) T cells and exogenously infected allogeneic CD4\(^+\) T cells. This assay can be used to monitor the level of antiviral activity of CD8\(^+\) T cells in a retrospective and prospective manner in studies of the natural history of HIV-1 infection and of subjects receiving anti-HIV-1 therapy and vaccines.

Infection with human immunodeficiency virus type 1 (HIV-1) results in a persistent decline in numbers of CD4\(^+\) T lymphocytes and degree of cellular immune function. This leads to development of AIDS in an average of 10 years in adults (11, 15). Host factors that control HIV-1 replication and disease progression are poorly defined.

Two major types of CD8\(^+\) T-cell-mediated anti-HIV-1 responses have been described in infected subjects. Cytotoxic T lymphocytes (CTL) function by lysis of HIV-1-expressing targets in a major histocompatibility complex (MHC) class I-restricted manner (14, 16, 18, 19). Koup et al. (10) have provided evidence for the protective capacity of CTL in that there is a decline in viral load during primary infection associated with an increase in levels of anti-HIV-1 memory CTL. Recently, we have reported that high-anti-HIV-1 CTL memory responses and low viral load are associated with long-term nonprogression in HIV-1-infected adults (17).

A second mechanism of cellular immunologic control of HIV-1 replication is via a noncytolytic, non-MHC-restricted mechanism mediated by a soluble factor secreted by CD8\(^+\) T cells (2, 12, 20, 21, 23). This HIV-1-suppressive activity has been detected in HIV-1-infected humans and has been correlated with disease status (15). A similar kind of antiviral activity has also been demonstrated with simian CD8\(^+\) T cells against simian immunodeficiency virus-infected CD4\(^+\) T cells from infected monkeys (5, 9). Assays of CD8\(^+\) T-cell-mediated HIV-1 suppression, however, are usually not quantitative and are highly variable. In this report, we describe a reproducible, quantitative HIV-1 suppression assay that can be used with a relatively small number of both fresh and cryopreserved peripheral blood mononuclear cells (PBMC).

**MATERIALS AND METHODS**

**Subjects.** HIV-1-seropositive subjects were recruited from the Pittsburgh portion of the Multicenter AIDS Cohort Study. They were divided into three groups by the slope of the numbers of peripheral blood CD4\(^+\) T cells over approximately 10 years: the stable group included two subjects with a stable or increasing slope of CD4\(^+\) T cells (slope, –2 to +9 cells/mm\(^3\) per 6 months); the intermediate group included three subjects with a moderate decline in the slope of CD4\(^+\) T cells (slope, –30 to –49 cells/mm\(^3\) per 6 months); the rapid group included three subjects with a fast decline in CD4\(^+\) T cells (slope, –83 to –166 cells/mm\(^3\) per 6 months) (8). The stable and intermediate groups were asymptomatic. One of the three rapid group subjects had AIDS as determined by the 1987 Centers for Disease Control definition. Blood was used from the subjects of all three groups at later time points (during 1995 to 1996). At this time, mean CD4\(^+\) cell numbers were 493 (range, 437 to 549) for the stable group, 499 (range, 239 to 825) for the intermediate group, and 335 (range, 303 to 349) for the rapid group. At the time of collection, only two subjects (from the rapid group) were receiving antiviral therapy.

Blood from HIV-1-seronegative donors was provided by the Central Blood Bank, Pittsburgh, Pa.

**Separation of CD4\(^+\) and CD8\(^+\) T cells.** PBMC were separated from whole blood on Ficoll-Hypaque gradients, washed two times with Hank’s balanced salt solution (HBSS), and resuspended in culture medium consisting of RPMI 1640 medium with 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) and 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS). Purification of CD4\(^+\) and CD8\(^+\) lymphocytes from PBMC was done by attachment to anti-CD4 or anti-CD8 microCelector flasks (AIS, Menlo Park, Calif.) according to the manufacturer’s directions as described by Hausner et al. (7) and Ferbas et al. (6). Briefly, 30 × 10\(^6\) to 40 × 10\(^6\) PBMC in 6 ml of the culture medium were incubated in an anti-CD4 flask at room temperature for 1 h. The nonadherent cells were removed and incubated in an anti-CD8 flask for 1 h at room temperature for CD8\(^+\) cell separation. Following incubation, the nonadherent cells were removed from the anti-CD8 flasks. The anti-CD4 or anti-CD8 flask was washed five times with HBSS, and the adherent cells were incubated in stimulation medium containing RPMI 1640 medium with 20% FCS, 10% normal human interleukin-2 (Cellular Products, Buffalo, N.Y.), 200 U of recombinant human interleukin-2 (Cetus Corp., Emeryville, Calif.) per ml, 200 ng of anti-CD3 monoclonal antibody (mAb) (Ortho Diagnostics, Raritan, N.J.) per ml, and 25 mM HEPES for 3 to 6 days. Following incubation, adherent cells from the anti-CD4 flask and the anti-CD8 flask were removed from the surface of the flask and pelleted at 1,000 × g for 10 min. The purity of the CD4\(^+\) and CD8\(^+\) T-cell populations was routinely greater than 90% as determined by flow cytometry (16). The CD4\(^+\) and CD8\(^+\) T cells were then used for the suppression assay.

The anti-CD4 and anti-CD8 flasks were found to be inefficient for the purification of the CD4\(^+\) and the CD8\(^+\) T cells from less than 10 × 10\(^6\) frozen-thawed PBMC. Therefore, CD4\(^+\) and CD8\(^+\) T cells from relatively small numbers of frozen-thawed PBMC were separated by use of anti-CD4 or anti-CD8 monoclonal antibody-coated immunomagnetic (IM) beads (Dynabeads M-450; Dynal, Oslo, Norway) as described in the manufacturer’s directions. Briefly, CD4 IM beads were washed three times with cold phosphate-buffered saline (PBS) containing 2% FCS (PBS-2% FCS) and incubated with the frozen-thawed PBMC at a bead-to-target-cell ratio of 5:1 in cold PBS-2% FCS at 4°C on a rotating- rocking platform for 1 h. The cells rosetted with IM beads were then isolated by placing the tube in a magnetic device (Dynal magnetic particle concentration) for 1 to 2 min followed by removal of the supernatant containing unbound cells. The rosetted cells were washed four times with cold PBS-2% FCS and mixed with 100 μl of a goat anti-mouse Fab reagent (Detaehabead; Dynal). The mixture was agitated at room temperature for 1 h. Bead-free pure CD4\(^+\) T cells were separated by placing the tube in the magnetic device for 1 to 2 min and collecting the supernatant. The unbound (CD4-depleted) cells were then used for CD8\(^+\) T-cell
purification by binding with anti-CD8 IM beads followed by treatment with Detachabead by the same procedure as that described for the CD4⁺ purification. The purity of CD4⁺ and CD8⁺ T cells prepared in this way was >90% as assessed by flow cytometry. The purified CD4⁺ T cells were cultured for 5 days in stimulation medium and then used in the suppression assay.

Preparation of endogenously infected target cells. To maintain relatively comparable levels of viral load in the HIV-1 suppression assays, all endogenously infected CD4⁺ T cells were pretitrated for HIV-1 levels before the suppression assay. Purified CD4⁺ T cells from HIV-1-seropositive subjects were frozen in aliquots in 10% dimethyl sulfoxide at −135°C (8). Viral load was determined by culturing a fivefold serial dilution of a frozen-thawed aliquot of CD4⁺ T cells in 24-well plates in the stimulation medium without anti-CD3 MAb for 10 days. Replication of HIV-1 was determined by p24 measurement using an antigen capture assay (Dupont, Boston, Mass.).

Preparation of exogenously infected CD4⁺ target cells. CD4⁺ T cells were purified from the blood of HIV-1-seronegative controls with the anti-CD4 flasks as described above. Forty million CD4⁺ T cells were incubated in RPMI 1640 medium containing 5 μg of Polybrene per ml for 3 h at 37°C. The cells were subsequently pelleted and resuspended in 1 ml of HIV-1 strain BRU (400,000 pg of p24 per ml). The cells were incubated with virus for 1 h at 37°C, after which they were washed twice with HBSS and cultured in the stimulation medium (without anti-CD3 MAb) for 24 h. Following incubation, the infected cells were frozen in RPMI 1640 medium with 10% FCS and 10% dimethyl sulfoxide at −135°C. An aliquot of the frozen HIV-1 strain BRU-infected cells was thawed and used for viral load measurement by culture as described above for endogenously infected CD4⁺ T cells.

HIV-1 suppression assay. A schematic diagram of the suppression assay, which is a modification of the assay of Ferbas et al. (6), is presented in Fig. 1. The minimum number of endogenously infected or HIV-1 BRU-infected CD4⁺ T cells that produced 1,000 to 5,000 pg of HIV-1 p24 during the 10 days of culture was used as a target for the assay. In this way, a large number of aliquots of cryopreserved CD4⁺ T cells with a predetermined viral load could be used for many experiments. For the suppression assay, an aliquot of CD4⁺ T cells (usually 10,000 to 20,000 HIV-1 BRU-infected cells and 30,000 to 100,000 endogenously infected cells) was cultured alone or cocultured with five fivefold serial dilutions of CD8⁺ T cells starting with 5 × 10⁶ cells in a 24-well plate containing 1 ml of stimulation medium (without anti-CD3 MAb) for 10 days. Viral replication was monitored by measuring p24 in the culture medium.

To perform the assay in a transwell plate (Costar, Cambridge, Mass.), the assay was done as described above for the mixing experiment except that the CD4⁺ T cells were placed in the top chamber and the CD8⁺ T cells were placed in the bottom chamber of the transwell plate. Following 10 days of culture, supernatant was collected from the top chamber and used for p24 measurement. To measure the HIV-1-suppressive activity in supernatant from the CD8⁺ T cells, the purified CD8⁺ T cells were incubated in stimulation medium for 6 days at 3 × 10⁶ cells/ml. Culture fluids were collected and stored at −70°C until used. The infected CD4⁺ T cells were cultured in thawed supernatants for 10 days. The supernatant was then collected and tested for p24.

Suppression levels were expressed as the percentage of inhibition of p24 production (picograms of p24 in cultures with HIV-infected targets and CD8⁺ T cells or supernatant/picograms of p24 in cultures with target cells only) × 100]. Suppression units per million CD8⁺ T cells were calculated by dividing one million by the minimum number of CD8⁺ T cells which inhibited 50% p24 production by infected CD4⁺ T cells cultured alone.

**RESULTS**

Measurement of the level of CD8⁺ cell suppressive activity. CD8⁺ T cells isolated from HIV-1-seropositive men were stimulated with anti-CD3 MAb for 3 to 5 days. They were then tested for HIV-1-suppressive activity by mixing with either autologous in vivo naturally infected CD4⁺ T cells or allogeneic CD4⁺ T cells from a seronegative donor that were infected in vitro with HIV-1 strain BRU. Figure 2 shows representative results of various degrees of suppressive activity of CD8⁺ T cells from three HIV-1-seropositive subjects when endogenously infected autologous CD4⁺ T cells (Fig. 2A) and allogeneic cells infected in vitro with BRU (Fig. 2B) were used. In general, the level of HIV-1-suppressive activity with in vivo HIV-1-infected autologous CD4⁺ T cells was comparable to that with exogenously HIV-1 BRU-infected allogeneic CD4⁺ T cells.

The level of HIV-1-suppressive activity was also tested in transwell chambers in which CD8⁺ T cells and HIV-1-infected CD4⁺ T cells (autologous or allogeneic) were separated by a
membrane. Figure 3A shows representative results from a stable group subject with a CD4\(^+\) cell number of 549. HIV-1-suppressive activity of CD8\(^+\) T cells was comparable to that of mixtures of endogenously infected autologous CD4\(^+\) T cells and CD4\(^+\) cells separated in the transwells. However, HIV-1-suppressive activity of CD8\(^+\) cells of the same subject against exogenously infected allogeneic cells was observed predominantly when CD8\(^+\) and CD4\(^+\) T cells were mixed together and not in the transwell system (Fig. 3B). Similar results were obtained from three more subjects (data not shown). The culture supernatants from CD8\(^+\) T cells from two seropositive group 2 subjects with CD4\(^+\) T-cell counts of 395 and 239, respectively, were also tested for HIV-1-suppressive activity (Fig. 4). Although potent anti-HIV-1 activity was demonstrated in the culture supernatant against autologous HIV-1-infected CD4\(^+\) T cells, the same supernatant from the CD8\(^+\) T cells showed poor suppressive activity against BRU-infected allogeneic CD4\(^+\) T cells. Similar discordant antiviral activity was observed with supernatant from activated CD8\(^+\) cells from five more subjects (data not shown).

The validity of the suppressive activity of the CD8\(^+\) T cells in the cell mixtures was examined by split-sample testing of three aliquots of the same blood sample for suppressive activity of CD8\(^+\) T cells of a seropositive subject against HIV-1 BRU-infected allogeneic CD4\(^+\) T cells. Table 1 shows that suppressive activities were highly comparable between samples. We have also examined the suppression activity in fresh PBMC of a subject with stable CD4\(^+\) T cells at two time points 1 month apart. The results were very similar (526 suppression units/10\(^6\) CD8\(^+\) cells versus 1,250 suppression units/10\(^6\) CD8 cells).

CD8\(^+\) cells suppressive activity was not due to cytolytic activity. To determine whether HIV-1-suppressive activity in the cell mixtures was due to cytolytic activity, we performed the suppression assay with allogeneic CD4\(^+\) T cells from two HIV-1-seropositive subjects that were mismatched at all MHC class I loci with the CD8\(^+\) effector T cells. Figure 5 shows that the suppression of HIV-1 replication in autologous CD4\(^+\) cells was comparable to that obtained with in vivo HIV-1-infected MHC class I mismatched allogeneic CD4\(^+\) T cells.

To further delineate that the HIV-1-suppressive activity in cell mixtures was not mediated through cytolytic activity, CD8\(^+\) T cells were cultured with in vivo-infected, autologous CD4\(^+\) cells. After 7 days, when suppression of HIV-1 replication was observed, CD4\(^+\) T cells were separated from the mixture and cultured alone. The results shown in Fig. 6 indicate that the CD4\(^+\) T cells after separation were capable of supporting endogenous HIV-1 replication, indicating that the cells were not lysed. As a control, HIV-1 replication remained suppressed in an unseparated CD8\(^+\) and CD4\(^+\) T-cell mixture.

Comparison of HIV-1-suppressive activity between fresh and frozen-thawed CD8\(^+\) T cells. CD8\(^+\) T cells were purified from fresh PBMC of a seropositive subject by use of the anti-CD8 flask as described in Materials and Methods. An aliquot
of fresh CD8+ T cells was stored at −135°C for 4 to 8 weeks. The HIV-1-suppressive activity of fresh and frozen-thawed CD8+ T cells from this subject was compared against three different HIV-1-infected CD4+ T cells as targets, i.e., endogenously infected autologous CD4+ T cells (Fig. 7A), endogenously infected allogeneic CD4+ T cells (Fig. 7B), and allogeneic CD4+ T cells from a seronegative donor that were exogenously infected with HIV-1 BRU (Fig. 7C). The suppressive activity of fresh CD8+ T cells was comparable to or slightly higher than that obtained with frozen-thawed CD8+ T cells regardless of the source of the HIV-1-infected CD4+ target cells. These results further support the reproducible nature of the assay.

Finally, we compared the HIV-1-suppressive activity of CD8+ T cells isolated from fresh PBMC with that from frozen-thawed PBMC from two HIV-1-seropositive subjects (Fig. 8) against allogeneic CD4+ T cells exogenously infected with HIV-1 BRU. As shown in Fig. 8, the antiviral activity of CD8+ T cells from frozen-thawed PBMC was comparable to that obtained from fresh PBMC.

Measurement of the suppressive activity of CD8+ cells in subjects with different degrees of CD4+ T-cell decline. Eight HIV-1-seropositive and four HIV-1-seronegative subjects were used in this study. Of the eight seropositive subjects, two had a stable or increasing slope of CD4+ T cells over a period of 9.5 years, while three had intermediate and three had sharp decreases in the slope of CD4+ T cells during the same period of time. As shown in Fig. 9, high levels of HIV-1-suppressive activity were detected in the three subjects with a stable slope of CD4+ T cells. In contrast, the levels of HIV-1-suppressive activity in subjects with an intermediate decline in CD4+ T cells were variable, and that in the subjects that fast decline were extremely low. HIV-1-suppressive activity was not detectable in CD8+ cells from the four HIV-1-seronegative subjects.

**TABLE 1. Validity of the HIV-1 suppression assay by CD8+ T cells**

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<th>No. of CD8+ T cells</th>
<th>HIV-1 suppression (%)</th>
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<td>0.5 × 10^6</td>
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<td>1 × 10^6</td>
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<td>2 × 10^6</td>
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<td>4 × 10^6</td>
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<td>8 × 10^2</td>
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* CD8+ T cells from a seropositive subject was tested in three aliquots (I to III) in a single experiment for HIV-1-suppressive activity against HIV-1 BRU-infected CD4+ T cells from a seronegative subject.
DISCUSSION

This report describes a semiquantitative assay for suppression of HIV-1 replication in CD4\(^+\) T cells by CD8\(^+\) lymphocytes. The assay has several characteristics that make this method most suitable for the measurement of CD8\(^+\) T-cell-mediated antiviral suppression. First, the assay provides a quantitative measure of the suppressive activity. Second, the assay has a wide dynamic range that will be extremely useful in monitoring the level of suppressive activity of CD8\(^+\) T cells against HIV-1 infection. This has been illustrated by a 650-fold difference in suppressive activity between subjects with stable CD4\(^+\) T-cell numbers and that of subjects with a rapid decline in CD4\(^+\) T-cell numbers. Third, the assay uses a relatively low number of either freshly donated or frozen-thawed CD4\(^+\) T cells (1 \(\times\) 10\(^5\) to 5 \(\times\) 10\(^5\)) and CD8\(^+\) T cells (0.15 \(\times\) 10\(^6\) to 0.625 \(\times\) 10\(^6\)) and therefore can be applied to subjects where the number of PBMC is limited, such as AIDS patients and HIV-1-infected infants. Fourth, the assay utilizes CD4\(^+\) T cells containing a fixed amount of viral load. The assay can consequently be used effectively to measure HIV-1-suppressive activity in longitudinal PBMC samples obtained at different stages of the disease, where HIV-1 load changes dramatically as the disease progresses. Although the antiviral activity against endogenously infected autologous CD4\(^+\) cells is the preferred assay, an assay using exogenously infected CD4\(^+\) T cells may be more useful in subjects with very low viral load such as those having received potent antiretroviral therapy or at the early stages of HIV-1 infection.

Our assay is similar to that described by Ferbas et al. (4). However, their method is not quantitative because it uses a percentage of inhibition of the p24 readout system from a single well with CD4\(^+\) cells with pretitrated viral load and CD8\(^+\) cells, which has a low dynamic range (10-fold). The assays described by Mackewicz et al. (13) and Hausner et al. (7) are claimed to be semiquantitative. However, because of their extremely low dynamic range (50-fold for the assay described by Mackewicz et al. [13] and 8-fold for the assay described by Hausner et al. [7]), these assays cannot be suitably used to measure changes in antiviral activity during disease progression or following antiviral therapy. Furthermore, both of these latter assays use 4 \(\times\) 10\(^5\) to 6 \(\times\) 10\(^5\) CD4\(^+\) cells and 2 \(\times\) 10\(^6\) to 4 \(\times\) 10\(^6\) CD8\(^+\) cells, which is much higher than that required in our assay. This could be very important when the number of PBMC is limited. These two assays also do not use CD4\(^+\) cells with a fixed amount of viral load and therefore could yield results that could be subjected to misinterpretation during a longitudinal study in which viral load changes significantly as disease progresses.

Our assay performed best when CD8\(^+\) T cells were mixed with CD4\(^+\) T cells regardless of whether the target cells were in vivo-infected, autologous CD4\(^+\) T cells or allogeneic CD4\(^+\) T cells infected in vitro with HIV-1 strain BRU. The antiviral suppression observed was not due to cytolytic activity because (i) the HIV-1-suppressive activity was still detected when CD4\(^+\) and CD8\(^+\) T cells were separated by a semipermeable membrane and was also mediated by supernatant from the CD8\(^+\) T cells, (ii) the HIV-1-suppressive activity was not MHC class I restricted, and (iii) HIV-1 replication in CD4\(^+\) T cells was restored by removing CD8\(^+\) T cells from the mixture. It is not clear why supernatant from CD8\(^+\) T cells, or CD8\(^+\) T cells in the transwell system, suppressed HIV-1 replication in endogenously infected autologous CD4\(^+\) T cells but less so in allogeneic cells exogenously infected with HIV-1 BRU. As men-
tioned earlier, this apparent discrepancy was not due to MHC class I restriction. It is possible that there are other MHC alleles that are important for the mediation of antiviral suppressor activity. However, it should be pointed out that only HIV-1 BRU-infected cells were used as targets in the suppression assay by supernatant or in the transwell system. In most studies, supernatant was tested against HIV-1 strains other than BRU. Therefore, it is possible that the apparent discrepancies could be due to the difference in HIV-1 strains used in the study. This study needs to be extended to test other HIV-1 strains, particularly clinical isolates, as targets in the suppression assay. Cocchi et al. (4) have recently reported that CD8+ cell-mediated HIV-1 suppression is due to the chemokines RANTES, MIP-1β, and MIP-1β. However, in our hands, antibodies to these chemokines either alone or in combination did not block suppressive activity of CD8+ T cells against either endogenously infected autologous CD4+ T cells or exogenously infected allogeneic CD4+ T cells (3). These results indicate that the antiviral activity of CD8+ T cells presented here was not mediated through these three chemokines.

The flexibility of our assay was demonstrated by its ability to perform equally well with both fresh and frozen-thawed CD8+ T cells, regardless of the source of HIV-1-infected CD4+ T cells. Recently, we have compared the suppressive activity of CD8+ cells from frozen PBMC of four subjects with stable or slow decline in CD4+ cells from the Multicenter Aids Cohort Study that have been stored at −135°C for 1 to 3 years with that of their fresh PBMC. Comparable suppressive activity (within fivefold) was noted in most cases (unpublished data). Data presented here indicate that the HIV-1-suppressive activity was detected only in CD8+ T cells from HIV-1-seropositive subjects and not in those from healthy HIV-1-seronegative subjects. Detection of antiviral activity by CD8+ T cells from healthy seronegative subjects is controversial. Brinchmann et al. (2) and Bagasra and Pomerantz (1) have reported low levels of antiviral activity in CD8+ T cells from seronegative subjects. Similarly, Mackewicz et al. (13) also showed suppressive activity, albeit at a low level, in CD8+ cells from HIV-1-seronegative subjects. In contrast, Walker et al. (22) have reported that CD8+ cells from HIV-1-seronegative subjects are unable to inhibit HIV-1 replication. Levy et al. (12) mentioned that this suppressive activity appears to be linked to previous exposure to HIV-1 and perhaps to another lentivirus. It appears, therefore, that activated CD8+ T cells from HIV-1-seronegative subjects either do not have suppressive activity or have a very low level of antiviral activity.

The potential use of the HIV-1 suppression assay described in this report has been demonstrated by examining the level of antiviral activity of CD8+ T cells from subjects at different stages of disease as defined by different slopes of decline in CD4+ T-cell numbers. These data clearly showed a correlation between the degree of antiviral activity of CD8+ T cells and the stage of disease. Our results support the concept that suppression of HIV-1 replication by CD8+ T cells may be important in the host response to this virus.

In summary, the CD8+ T-cell-mediated HIV-1 suppression assay described in this report is reproducible, has a wide dynamic range, and can be applied to relatively small numbers of fresh and frozen-thawed PBMC. We previously showed that fresh and cryopreserved PBMC can be used to assess anti-HIV-1 memory CTL responses (8). Therefore, these two assays can now be applied to nonconcurrent prospective studies of both anti-HIV-1 CD8+ T-cell suppression and CTL activities in natural history studies and in subjects receiving antiviral therapy and vaccines.

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