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# Suppression of Human Immunodeficiency Virus Type 1 Replication by CD8$^+$ Cells: Evidence for HLA Class I-Restricted Triggering of Cytolytic and Noncytolytic Mechanisms

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**Although CD8$^+$ lymphocytes in human immunodeficiency virus type 1 (HIV-1)-infected individuals have been demonstrated to suppress viral replication, the mechanisms of inhibition have not been defined precisely. A large body of evidence indicates that these cells act via soluble inhibitory factors, but the potential role of HLA class I-restricted cytolysis has remained controversial. Here we demonstrate that HIV-1-specific cytotoxic T lymphocytes (CTL) mediate antiviral suppression by both cytolytic and noncytolytic mechanisms. The predominant mechanism requires direct contact of CTL with the infected cells, is HLA class I restricted, and can achieve complete elimination of detectable virus in infected cell cultures. Inhibition occurs even at high multiplicities of infection or at ratios of CTL to CD4 cells as low as 1:1,000. The other mechanism is mediated by soluble inhibitory factors which are triggered in an antigen-specific and HLA-restricted fashion but then act without HLA restriction. These include MIP-1α, MIP-1β, and RANTES, as well as a distinct factor(s) capable of inhibiting HIV-1 strains insensitive to these chemokines. These data indicate that HIV-1-specific CTL are potent mediators of HIV-1 suppression at cell ratios existing in vivo and demonstrate an antigen-specific trigger for CD8$^+$ cell-derived soluble inhibitory factors. These results suggest that CTL play an important role in the observed antiviral activity of CD8$^+$ cells from infected individuals.**

HLA class I-restricted cytotoxic T lymphocytes (CTL) are a subset of CD8$^+$ lymphocytes which have been shown to play a crucial role in various viral infections (45, 49, 68). Abundant clinical evidence suggests that CTL are a key protective immune response in human immunodeficiency virus (HIV-1) infection as well. Virus-specific CTL have been identified in exposed but uninfected individuals (37, 48, 51), and antiviral cytotoxic activity is correlated temporally with the clearance of viremia in primary infection (5, 33). Decline in this antiviral response coincides with disease progression in infected individuals (7, 31), and long-term nonprogressing seropositive individuals have been described to have vigorous and broadly directed HIV-1-specific CTL activity (15, 19, 20, 50). Most of these studies have examined the presence of CTL by using chromium release assays against target autologous B cells expressing recombinant HIV-1 antigens, and there are few data regarding the interaction of CTL with HIV-1 infected cells. The CTL present in HIV-1-infected individuals have therefore been well defined, although functional data concerning their effects on viral replication have been lacking.

In contrast, CD8$^+$ cells from infected individuals clearly have been shown to suppress HIV-1 replication in autologous CD4$^+$ cells (6, 8, 40, 56, 61, 65), but the mechanisms of this effect have remained controversial. Specifically, the relationship of CTL to the CD8$^+$ effector cells has remained unclear (57). Since the initial observation that CD8 cells are capable of inhibiting HIV-1 replication by noncytolytic mechanisms (61), numerous investigators have proposed that CD8$^+$ CTL do not account for the observed antiviral effect of CD8$^+$ cells. The major arguments for this viewpoint include (reviewed in reference 38) the demonstration that CD8$^+$ cells can inhibit HIV-1 across a semipermeable membrane via soluble factors, the ability of bulk CD8$^+$ cells to mediate viral inhibition without HLA restriction, the reversibility of inhibition by CD8$^+$ cells, and the relatively low ratios of CTL presumed to be present in vivo. However, the interpretation of many studies addressing these issues is limited by the use of mixed effector cell populations. In addition, most previous studies of CD8$^+$ cell-derived soluble inhibitory factors have utilized mitogen-stimulated cells, and the triggering requirements for release of these factors therefore remain undefined.

In this study, we performed a detailed analysis of the ability of HIV-1-specific CD8$^+$ CTL to inhibit HIV-1 replication by cytolytic and noncytolytic mechanisms. The effector cells were cloned CTL of defined epitope specificity and HLA restriction. To determine the role of HLA molecules, we used HIV-1-permissive target cell lines of known HLA types, including cells transfected with a defined HLA molecule. Our results indicate that CTL inhibit HIV-1 replication in a class I-restricted fashion and that this recognition event is the trigger for target cell lysis and concomitant release of soluble mediators of viral suppression.

## MATERIALS AND METHODS

**HIV-1-permissive target cell lines. (i) Immortalized HIV-1 permissive cell lines.** T1 (HLA A2, B5) (52), H9 (HLA A1, B6, Bw62, Cw3), H9-B14 (H9 cells stably transfected with class I HLA B14 cDNA), and PM1 (39) cells were maintained in RPMI 1640 (Sigma, St. Louis, Mo.) supplemented with 20% heat-inactivated fetal calf serum (FCS; Sigma), 10 mM HEPES, 2 mM glutamine, 100 U of penicillin per ml, and 10 μg of streptomycin per ml (R20). H9-B14 cells were generated as previously described (67). Near-confluent cells were split 1:2 in fresh medium the day before infection.

**(ii) Bulk CD4-positive cell line from an HIV-1-seronegative individual.** Polyclonal CD4$^+$ cells (greater than 98% CD3 and CD4 expressing by fluorescence-activated cell sorting analysis) were generated from seronegative individuals by

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the addition of CD3:CD8-bispecific monoclonal antibody and irradiated allogeneic feeder cells to peripheral blood mononuclear cells (PBMC) as previously described (64) without antiretroviral drugs. These cells were maintained in RPMI 1640 containing 10% heat-inactivated FCS, 10 mM HEPES, 2 mM glutamine, 100 U of penicillin per ml, 10 μg of streptomycin per ml, and 50 U of interleukin-2 (IL-2) per ml (R10-50) and restimulated at 2-week intervals with the anti-CD3 antibody 12F6 (66) and irradiated allogeneic feeder cells. The CD4 cells were infected 1 week after restimulation. A control $CD8^+$ cell line was generated and maintained in the same manner with a CD3:CD4-bispecific antibody (66).

**Virus stocks.** The T-lymphocytotropic (T-tropic) HIV-1 IIIB was taken from the supernatant fluid of freshly infected H9 cells. The viral titer (50% tissue culture infective doses [$TCID_{50}$] per milliliter) was determined by using C8166 cells as previously described (27). The monocytotropic (M-tropic) HIV-1 JR-CSF (35) was produced and subjected to titer determination by infecting phtohemagglutinin-stimulated PBMC (16). Both stocks were stored at −80°C until use.

**Effector cells. (i) CTL clones from HIV-1-infected individuals.** HIV-1 specific CTL clones were obtained by cloning of stimulated PBMC at limiting dilution and characterized for specificity and HLA restriction as previously described (26, 29, 58). The HLA A2-restricted CTL clones were 18030D23 (designated Gag/A2), specific for an HIV-1 Gag epitope (amino acids [aa] 77 to 85; SLYNTVATL), and 68A62 (designated RT/A2), specific for a reverse transcriptase (RT) epitope (aa 476 to 484; ILKEPVHGV). HLA B14-restricted clones included 115M21 (designated Env/B14), recognizing a gp41 epitope (aa 584 to 592; ERYLKDQQL), and 15160A49 (designated Gag/B14), recognizing a Gag epitope (aa 298 to 306; DRFYKTLRA). An HLA A11-restricted clone 63D35 (designated RT/A11) recognized HIV-1 RT (aa 313 to 324; AIFQSSMTK). Amino acids are numbered according to the HXB2 sequence. CTL clones specific for hepatitis C virus were also obtained as previously described (36). All CTL clones were maintained in R10-50 and restimulated at least 10 days prior to use with irradiated allogeneic PBMC and 12F6 (66) or peptide-sensitized irradiated autologous Epstein-Barr virus-transformed B cells.

**(ii) Bulk CD8 cells from an HIV-1-seropositive individual.** CD8 cells (greater than 98% CD3 and CD8 expressing by flow cytometric assessment) were generated by positive selection with anti-CD8-antibody-coated immunomagnetic beads (Advanced Magnetics, Cambridge, Mass.). PBMC were isolated from peripheral blood by Ficoll gradient centrifugation and then incubated with beads at a ratio of five beads per cell in RPMI–1% FCS (R1) at 4°C with gentle agitation for 30 min. The magnetic beads were then separated from the supernatant fluid and washed twice in R1 with a magnetic separator (Advanced Magnetics). After being washed, the cells were eluted from the beads overnight in R20 at 37°C. The remaining $CD8^+$ cells were then maintained in R10-50 and used within 7 days without any in vitro stimulation.

**Synthetic peptides.** Synthetic peptides corresponding to the HLA B14-binding HIV-1 epitopes 1280 (Env 584 to 592; ERYLKDQQL) and 125P (Gag 298 to 306; DRFYKTLRA) were synthesized as free acids (model 432A peptide synthesizer; Applied Biosystems, Foster City, Calif.). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethyl sulfoxide (Sigma) with or without 1 mM dithiothreitol (Sigma).

**Direct coculture of effector cells with acutely infected cells.** Target cells were incubated with HIV-1 IIIB at the specified multiplicities of infection (MOIs) ($10^{-2}$ to 4 $TCID_{50}$ per cell) for 4 h at 37°C. In preliminary experiments, bulk $CD8^+$-cell-depleted PBMC from an infected individual were used (without the addition of exogenous virus). The cells were then washed twice and plated at $5 \times 10^5$ cells per well in a 24-well plate (most coculture experiments) or $5 \times 10^4$ cells per well in a 96-well U-bottom plate (for effector-to-target cell variation and bulk $CD8^+$ cell coculture experiments) containing R10-50. Effector cells were added at a ratio of 1:1 or as otherwise specified. At 2- to 4-day intervals, the cocultures were fed by removing and replacing half of the supernatant with fresh R10-50. The removed supernatant fluid was cryopreserved for p24 antigen quantitation by standard quantitative HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (commercial kit; Dupont, Boston, Mass.).

**Transmembrane assay for inhibition.** CTL clones were stimulated in a manner previously described (6) and assayed for transmembrane inhibitory activity. H9 cells were acutely infected with HIV-1 IIIB at an MOI of $10^{-2}$ $TCID_{50}$/cell. A total of $5 \times 10^5$ cells were placed per well in a six-well plate (outer chamber) and overlaid with a 0.4-μm-pore-size semipermeable membrane insert (Millipore, Bedford, Mass.). Within the insert (inner chamber) were placed $1 \times 10^6$ CTL clones (RT/A11; HLA mismatched) and $3 \times 10^6$ goat anti-mouse immunoglobulin G-coated beads (Perseptive Diagnostics, Cambridge, Mass.) which had been previously saturated with the mouse-anti-human CD3 antibody 12F6 (66). Each chamber contained a total volume of 3 ml of R10-50. Controls included addition of the CTL directly to the infected cells in the outer chamber, CTL without beads in the outer chamber, and beads alone in the outer chamber. At 2- to 4-day intervals, 2 ml was removed from the outer chamber for p24 analysis and replaced with fresh medium.

**Generation of inhibitory supernatant from CTL. (i) Antigen specific.** The CTL clone Env/B14 was cocultured at $2.5 \times 10^5$ cells/ml with $7.5 \times 10^5$ autologous Epstein-Barr virus-transformed B cells labeled with cognate epitope peptide per ml. These B cells had been labeled with 100 μg of peptide 128O per ml, washed twice, and irradiated. After coculture in R10-50 for approximately 18 h, supernatant fluid was harvested and cryopreserved at −80°C for later use. Controls included supernatant fluids from B cells with cognate peptide and no CTL, as well as CTL exposed to B cells labeled with a control epitope in Gag presented by HLA-B14, 125P.

**(ii) CD3 activated.** CTL clones were cocultured at $2.5 \times 10^5$ cells/ml with $7.5 \times 10^5$ CD3-cross-linking beads/ml in R10-50. The beads were prepared as for the transmembrane experiment above. After coculture for approximately 18 h at 37°C, supernatant fluid was harvested and cryopreserved at −80° for later use. Control supernatant fluids included those from CTL not stimulated by beads and those from beads alone. Cryopreserved supernatant fluids from CTL clones which were stimulated or not stimulated by CD3 cross-linking were tested for concentrations of MIP-1α, MIP-1β, and RANTES by use of commercial quantitative ELISA kits (R&D Systems, Minneapolis, Minn.) as specified by the manufacturer.

**Assay for inhibition of HIV-1 replication by CTL supernatants or chemokines.** Target cells (H9 or PM1) were acutely infected with HIV-1 (IIIB or JR-CSF) at an MOI of $10^{-2}$ $TCID_{50}$ per ml and resuspended in R20. The cells were then plated at $5 \times 10^5$ per well in a 24-well plate. CTL supernatants were tested undiluted (for H9-IIIB) or at a final dilution of 1:2 (for PM1–JR-CSF). The chemokines MIP-1α, MIP-1β, and RANTES (R&D Systems) were also tested alone or in combination at the specified concentrations. In some experiments, a mixture of polyclonal neutralizing antibodies for MIP-1α, MIP-1β, and RANTES (R&D Systems) was added as well. Supernatant fluid (1 ml) was removed for p24 measurement every 2 to 4 days and replaced with medium supplemented with CTL supernatant fluid, chemokines, and/or antibodies as indicated.

**Coculture of mixed HLA matched and mismatched infected cells with CTL.** H9 and H9-B14 cells were acutely infected with HIV-1 IIIB at an MOI of $10^{-2}$ $TCID_{50}$/cell for coculture with the Env/B14 CTL clone. Cultures of H9 alone, H9 plus CTL, H9-B14 alone, H9-B14 plus CTL, H9 plus H9-B14, H9 plus H9-B14 plus CTL, and H9 plus H9-B14 ($2.5 \times 10^5$) plus CTL were plated in total volumes of 2 ml of R10-50. Each culture contained $5 \times 10^5$ of each listed target and effector cell, except as specified (half the amounts). After 14 days of p24 monitoring, the cocultures indicated were CD8 cell depleted by resuspension in R1 with anti-CD8 antibody-coated magnetic beads at a bead to cell ratio of 100 to 1. After coincubation at 4°C with gentle agitation for 30 min, the supernatant fluid was bead depleted twice with a magnetic separator. The cells remaining in the supernatant fluid were then resuspended in R20 for further culture in a 24-well plate and p24 antigen monitoring. After $CD8^+$ cell removal, the remaining cells from the coculture of H9-B14 and Env/B14 CTL were 100% CD4 expressing by flow cytometric immunophenotyping (data not shown).

**Chromium release assay.** H9-B14 cells were acutely infected with HIV-1 IIIB at an MOI of 4 $TCID_{50}$/cell for use in chromium release assays as previously described (67). On day 5 after infection, these cells were >95% infected by flow cytometric assessment of intracellular p24 antigen (data not shown). At this time, H9, H9-B14, and the infected H9-B14 cells were chromium labeled for chromium release assays with added cognate peptide or chromium-unlabeled cells as indicated, at an effector-to-target-cell ratio of 5 to 1 (67).

## RESULTS

**CTL clones are potent inhibitors of HIV-1 replication in an HLA-restricted manner.** In preliminary experiments, we investigated the inhibitory activity of CTL by reconstituting endogenously infected, $CD8^+$ cell-depleted PBMC from infected individuals with autologous $CD8^+$ cells or HIV-1-specific CTL clones (data not shown). Autologous $CD8^+$ cells efficiently suppressed endogenous viral replication as previously reported (61), as did autologous CTL clones. However, breakthrough viral replication frequently occurred, reflecting in part endogenous viruses not recognized by the CTL clones (data not shown). Allogeneic HLA-mismatched CTL clones were variably inhibitory in this system. These data suggested more potent suppressive activity when effector and target cells were HLA matched, but interpretation was limited by the inability to control viral input and alloreactivity.

The above data indicated the need for a better-controlled system for the specific study of CTL antiviral activity. We therefore utilized acutely infected HIV-1-permissive immortalized cell lines with HIV-1-specific CTL clones isolated from infected individuals (67). Coculture of acutely HIV-1 IIIB-infected T1 cells (expressing HLA A2 but not B14) with A2-restricted Gag- or RT-specific CTL clones resulted in maximal suppression of HIV-1 replication by up to $10^5$-fold on day 8 (Fig. 1A). The Gag-specific CTL was reproducibly more inhibitory than the RT-specific CTL, correlating with previous find-

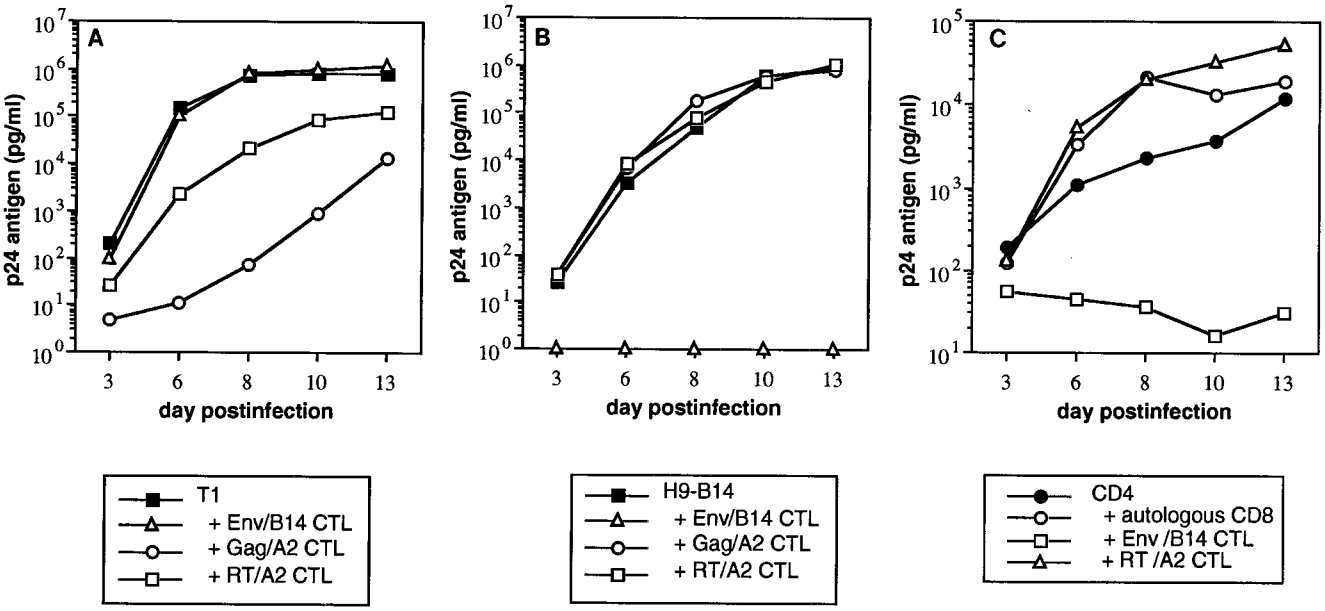


FIG. 1. Suppression of HIV-1 replication by CTL in direct coculture with infected CD4 cells. (A and B) T1 cells (HLA $A2^+/B14^-$) (A) and H9-B14 cells (HLA $B14^+/A2^-$) (B) were acutely infected with HIV-1 IIIB and cocultured with CTL clones specific for HIV-1 Gag (Gag/A2; HLA A2 restricted), RT (RT/A2; HLA A2 restricted), or Env (Env/B14; HLA B14 restricted) at a 1:1 ratio. Viral replication was assessed by serial quantitation of p24 antigen. Similar results were obtained upon repeat experiments and also with CTL from other individuals recognizing the same epitopes; all tested clones were suppressive (data not shown). (C) Primary $CD4^+$ cells from an HIV-1-seronegative donor who is HLA $B14^+/A2^-$ were infected with HIV-1 IIIB and cocultured with either autologous bulk $CD8^+$ cells or the CTL clone Env/B14 or Gag/A2 and assayed as above.

ings that A2-restricted Gag-specific CTL lyse infected T1 cells more efficiently than RT-specific CTL do (67). A B14-restricted Env-specific CTL clone, however, had no appreciable inhibitory effect on the infected T1 cells (Fig. 1A). In contrast, coculture of the same CTL clones with acutely infected H9 cells which had been transfected with the HLA B14 molecule (expressing HLA B14 but not A2, designated H9-B14) resulted in no inhibition by the A2-restricted CTL but greater than $10^5$-fold suppression by the B14-restricted Env-specific CTL clone (undetectable p24 by ELISA [Fig. 1B]). Furthermore, this B14-restricted clone did not inhibit infected H9 cells which lack the HLA B14 allele (see Fig. 6). CTL-mediated inhibitory activity was also verified on acutely HIV-1-infected primary $CD4^+$ lymphocytes from a seronegative donor who expresses HLA B14 but not A2. Autologous $CD8^+$ cells as well as an A2-restricted CTL clone had no inhibitory effect, whereas an Env-specific B14-restricted clone suppressed viral replication by $10^3$-fold (Fig. 1C).

**CTL clones are inhibitory at high viral input or at low effector concentrations.** Observations regarding viral dynamics and lymphoid reservoirs of HIV-1 suggest that relevant in vivo immune responses would need to function under conditions of high viral inocula. We varied the viral input of the above coculture system to evaluate the ability of CTL to suppress replication in cells infected with a higher viral burden. Coculture of CTL clones with HLA-matched target cells acutely infected at MOIs ranging from 0.02 to 4 $TCID_{50}$/cell yielded suppression of more than 99% even at the highest MOI (Fig. 2A and B), demonstrating vigorous inhibition at an inoculum previously determined to yield >98% infected cells (as assessed by intracellular p24 antigen immunostaining) by 4 days after infection (67).

We also determined the potency of this inhibition at limiting ratios of CTL to infected cells (Fig. 2C and D). Varying effector-to-target-cell ratios yielded inhibition even at initial ratios as low as one CTL clone per 1,000 $CD4^+$ cells. The Env-specific CTL clone used in this experiment (Fig. 2C) has been demonstrated by T-cell receptor analysis to represent 3% of the peripheral $CD8^+$ cells of the infected individual from whom it was isolated (28a), consistent with the frequencies of peripheral CTL clones described in another report (43). Bulk polyclonal $CD8^+$ cells from this individual (who is HLA A2 and B14 positive) were also able to inhibit acutely infected T1 and H9-B14 cells but not H9 cells, demonstrating the presence of relevant PBMC concentrations of A2- and B14-restricted CTL (Fig. 3). In sum, these data demonstrate the ability of CTL to inhibit HIV-1 replication despite high viral input and at effector cell concentrations comparable to those existing in vivo.

**Antigen-specific recognition triggers CTL to produce soluble HIV-1-inhibitory factors.** $CD8^+$ lymphocytes are known to produce soluble HIV-1-inhibitory factors (6, 8, 41, 56, 60), and CTL exert significant soluble antiviral activity in an experimental model of hepatitis B virus (18). We therefore investigated the contribution of soluble factors in the suppressive activity of CTL clones on HIV-1. CTL stimulated by CD3 cross-linking exerted moderate inhibition across a semipermeable membrane from HLA-mismatched acutely infected cells (Fig. 4A). The antigen specificity for release of the suppressive factors was demonstrated by culturing acutely infected cells in supernatant fluid from a CTL clone specifically stimulated with autologous B cells labeled with the CTL cognate epitope or with B cells labeled with an irrelevant peptide binding the same HLA molecule. The relevant target epitope was required to trigger CTL to produce soluble inhibitory factors (Fig. 4B). Of three CTL clones tested in four experiments by the transmembrane assay, all had activity, with a mean of 9.3% ± 5.1% transmembrane inhibition by unstimulated clones versus 55.0% ± 16.3% inhibition by CD3-cross-linked clones.

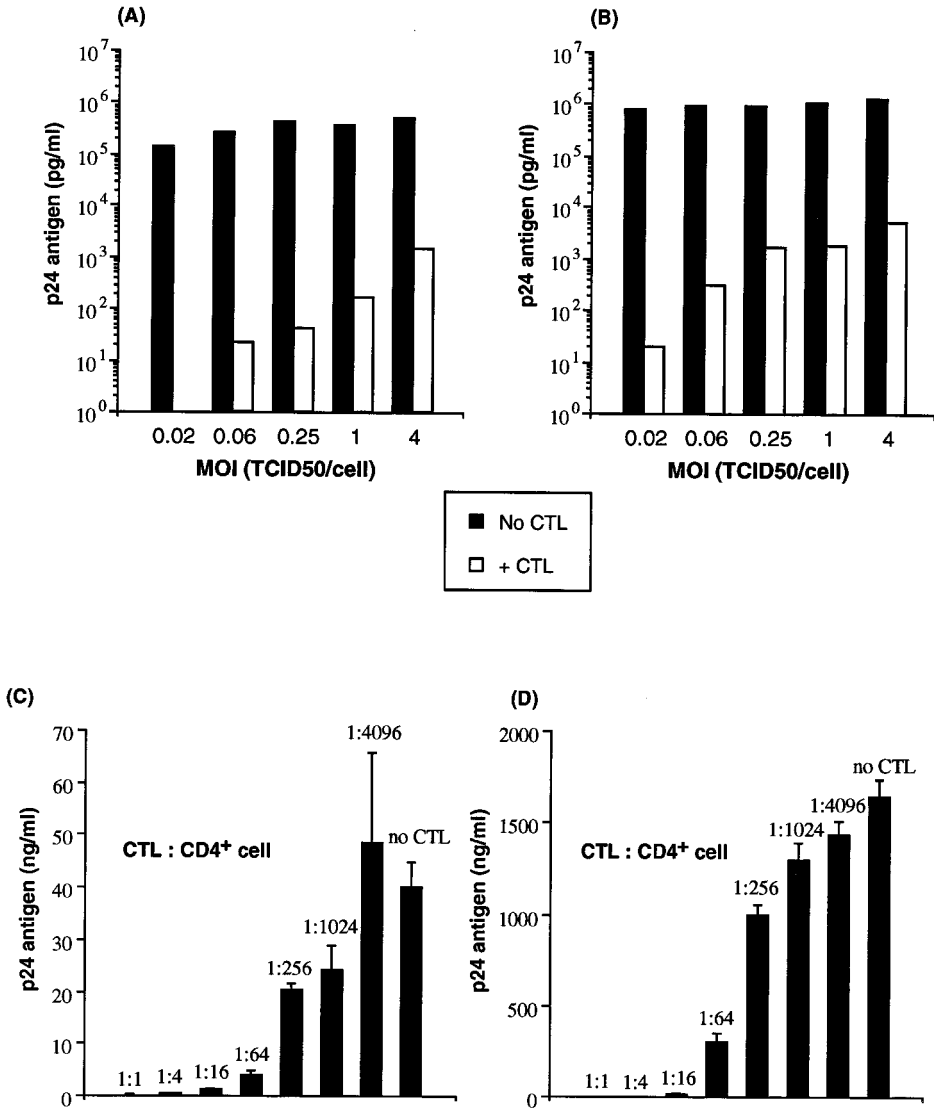


FIG. 2. Inhibition of HIV-1 replication by CTL under conditions of varying viral inputs and cell ratios. (A) H9-B14 cells were infected with HIV-1 IIIB at various MOIs ranging from 0.02 to 4 $TCID_{50}$/cell and cocultured at 1:1 with the CTL clone Env/B14. The day 7 p24 antigen concentration is shown. (B) T1 cells were infected and cocultured as in panel A with RT/A2. (C) H9-B14 cells were infected with HIV-1 IIIB at an MOI of $10^{-2}$ $TCID_{50}$/cell and cocultured with Env/B14 at various effector-to-target-cell ratios. Day 7 p24 antigen concentrations are shown, with standard error bars for quadruplicate samples. (D) T1 cells were infected and cocultured as in panel C with RT/A2.

**Activated CTL produce MIP/RANTES, as well as other inhibitory factors.** The chemokines MIP-1α, MIP-1β, and RANTES have been characterized as $CD8^+$ cell-derived soluble inhibitors of M-tropic strains of HIV-1 (9). We next assessed whether CTL produce these factors. Resting CTL clones produced very little of these substances (<2 ng/ml), but activation by CD3 cross-linking induced their production (Table 1). Evaluation of non-HIV-1-specific CTL also indicated that CTL with other specificities are capable of producing MIP or RANTES upon specific activation. This indicated that these C-C chemokines are among the soluble inhibitory factors produced by activated CTL. However, soluble factor-mediated inhibition of the T-tropic HIV-1 IIIB by CTL (Fig. 4) implied the presence of other suppressive factors. As previously described (9), MIP-1α, MIP-1β, and RANTES had no effect on HIV-1 IIIB replication in this system (alone or in combination [data not shown]). Furthermore, blocking antibodies to these chemokines did not ablate CTL soluble inhibitory activity on IIIB (data not shown).

We further evaluated inhibition by CTL supernatant fluid in a system analogous to that described by Cocchi et al. (9). In PM1 cells acutely infected with the M-tropic isolate HIV-1 JR-CSF, both supernatant and chemokines had a marked inhibitory effect (Fig. 5). A combination of MIP-1α, MIP-1β, and RANTES at 25 ng/ml each suppressed viral replication, and added CTL supernatant containing lesser amounts of these chemokines (Table 2 supernatant fluid from Gag/A2 clone; final culture concentrations: MIP-1α, 6 ng/ml; MIP-1β, 17 ng/ml; and RANTES, 2 ng/ml) was similarly inhibitory. Furthermore, blocking antibodies fully reversed the activity of the added chemokines but only partially offset the activity of the CTL supernatant fluid. This indicated that CTL also produce an inhibitory factor(s) distinct from MIP-1α, MIP-1β, and RANTES acting on JR-CSF, an M-tropic strain.

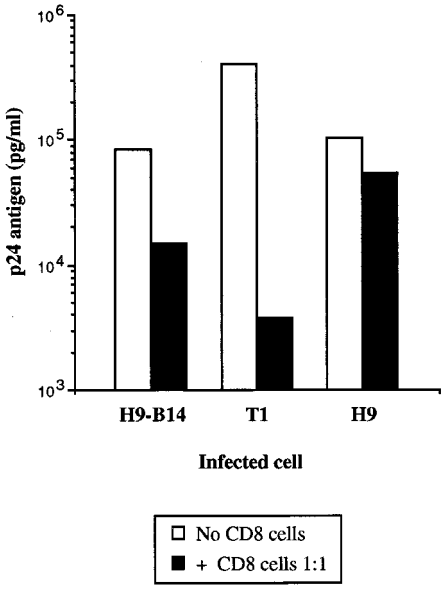


FIG. 3. Inhibition of HIV-1 by bulk $CD8^+$ lymphocytes from a seropositive individual. H9-B14, T1, and H9 cells were acutely infected with HIV-1 IIIB at $10^{-2}$ $TCID_{50}$/cell and cocultured at a 1:1 ratio with bulk $CD8^+$ cells from an HIV-1-infected individual. The H9-B14 and T1 cells each shared a class I HLA allele with the individual (HLA B14 and A2, respectively) whereas the H9 cells were entirely mismatched. The HIV-1 p24 concentration on day 7 is shown.

**The lytic component of CTL activity is required for potent and irreversible inhibition.** The above data indicated that infected H9 cells are not lysed by an HLA B14-restricted CTL clone whereas infected H9-B14 cells are lysed and also trigger the CTL to produce soluble inhibitory factors which act without HLA restriction. We used these properties to assess the relative contributions of cytolytic and noncytolytic antiviral activity by mixing HLA-matched and -mismatched infected target cells with a CTL clone. HIV-1 IIIB replication in HLA-mismatched H9 cells was not suppressed by coculture with an HLA B14-restricted CTL clone but was inhibited by the further addition of HLA-matched infected H9-B14 cells, which differed from H9 cells only by the restricting class I B14 molecule (Fig. 6A). At a 1:1 ratio of HLA-matched and -mismatched cells, replication was suppressed by 10- to 100-fold, whereas CTL inhibited replication in HLA-matched H9-B14 cells by more than $10^5$-fold (undetectable p24 by ELISA). The non-HLA-restricted inhibition was unlikely to be due to bystander lysis, since chromium-labeled infected H9 cells were not lysed when coincubated with infected H9-B14 cells and the B14-restricted CTL clone (Table 2). Subsequent removal of $CD8^+$ cells from the cocultures demonstrated that virus suppression in the coculture containing H9-B14 cells with CTL

TABLE 1. Production of MIP/RANTES by CTL clones[a]

| CTL clone | Concn (ng/ml) of: | | | | | |
|---|---|---|---|---|---|---|
| | RANTES | | MIP-1α | | MIP-1β | |
| | Unstim. | Stim. | Unstim. | Stim. | Unstim. | Stim. |
| Env/B14 | 1.3 | 11.3 | 1.1 | 23.7 | 0.4 | 44.5 |
| Gag/A2 | 0.3 | 4.1 | 0.2 | 12.0 | 0.2 | 34.7 |
| RT/A11 | 1.1 | 10.4 | 0.7 | 17.0 | 0.6 | 38.3 |
| HCV 94F P2-50 | 1.3 | 2.5 | 0.3 | 6.7 | 0.2 | 22.6 |
| HCV 93K.33 | 0.3 | 5.9 | 0.0 | 2.0 | 0.0 | 5.0 |

[a] CTL clones at $2.5 \times 10^5$ cells/ml were activated by CD3 cross-linking. For each clone, supernatant from stimulated (Stim.) or unstimulated (Unstim.) CTL was harvested after 18 h for ELISA quantitation of RANTES, MIP-1α, and MIP-1β.

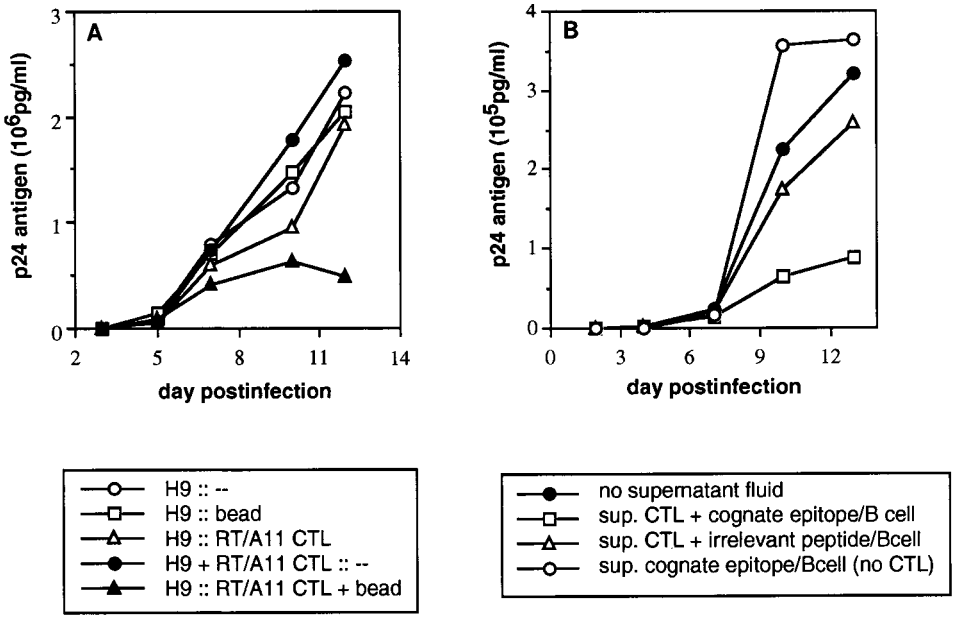


FIG. 4. Triggering of CTL to produce a soluble factor(s) which suppresses HIV-1 IIIB replication. (A) Transmembrane inhibition of HIV-1 replication was assessed by culturing H9 cells (negative for HLA A11) acutely infected with HIV-1 IIIB across a 0.4-μm-pore-size membrane from RT/A11 (an RT-specific, HLA A11-restricted CTL clone) which was activated by CD3 cross-linking. Controls included direct coculture of the H9 cells with RT/A11, transmembrane incubation with unstimulated RT/A11, and transmembrane incubation with CD3-cross-linking beads alone. Well contents are listed as outer chamber::inner chamber. (B) Inhibition of HIV-1 replication by supernatant fluid from CTL was assayed by culturing H9 cells acutely infected with HIV-1 IIIB in undiluted supernatant fluid from the CTL clone Env/B14, which was activated by exposure to irradiated autologous B cells labeled with the cognate Env epitope. Controls included supernatant fluid from B cells plus cognate peptide in the absence of CTL and supernatant fluid from Env/B14 exposed to B cells with an irrelevant Gag epitope binding HLA B14.

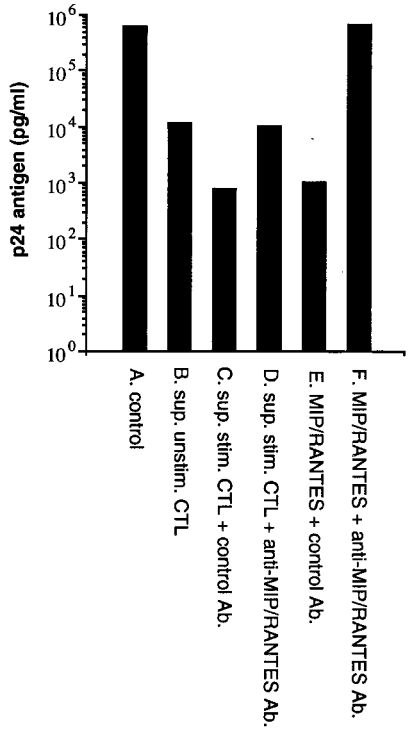


FIG. 5. Demonstration that CTL soluble-factor inhibition of HIV-1 JR-CSF is not limited to MIP/RANTES. PM1 cells acutely infected with the M-tropic strain HIV-1 JR-CSF were cultured in CTL supernatant fluid (from CD3-cross-linking-activated Gag/A2) at a 1:2 dilution or with a mixture of MIP-1α, MIP-1β, and RANTES at 25 ng/ml each (exceeding the MIP/RANTES concentration in the culture containing CTL supernatant fluid [Table 1]). Each test culture also contained control goat IgG (150 μg/ml) or a mixture of goat anti-human MIP-1α, MIP-1β, and RANTES antibodies (Ab.) (50 μg/ml each). Control cultures included infected cells alone, as well as supernatant fluid from unstimulated CTL, in the presence or absence of antibodies.

was irreversible (Fig. 6B), suggesting a lytic mechanism for inhibition in HLA-matched infected cells. This finding was in contrast to observations in other experimental systems that $CD8^+$ cell antiviral activity is reversible and noncytolytic (6, 41, 60, 65). Upon rechallenge with HIV-1 IIIB, the remaining $CD4^+$ cells from the H9-B14/CTL coculture were readily infected (Fig. 6C). In contrast, the uninfected $CD4^+$ cells continued to produce no detectable p24 antigen at 4 weeks after $CD8^+$ cell removal (Fig. 6C). These in vitro data demonstrate that CTL suppress viral replication more efficiently in HLA-matched cells than through noncytolytic effects on HLA-mismatched cells and that HLA-matched CTL can clear detectable infectious virus.

TABLE 2. Lack of HLA-mismatched bystander lysis of H9 cells[a]

| Chromium-labeled target cell | Added peptide or cell (no chromium) | % Specific lysis by Env/B14 |
|---|---|---|
| H9-B14 | Peptide epitope | 34 |
| Infected H9-B14 | None | 41 |
| H9 | Peptide epitope | 11 |
| H9 | Infected H9-B14 | 7 |

[a] Target cells were chromium labeled and used for chromium release assays with the B14-restricted CTL clone Env/B14. Unlabeled cells or the cognate epitope for the CTL were added to the target cells as indicated.

## DISCUSSION

HIV-1-specific CTL exert potent antiviral effects that are mediated by two distinct mechanisms. The first requires direct contact of the CTL with infected target cells, resulting in cytolysis; the second is mediated by soluble inhibitory factors produced by CTL. Both processes require antigen-specific, HLA-restricted activation through the T-cell receptor. The ability of CTL to completely and irreversibly inhibit HIV-1 replication suggests their ability to act within the narrow window (approximately 1 day) between cellular infection and virion production defined by studies of viral dynamics (21, 46, 63). This is consistent with our earlier finding that CTL can lyse infected cells early in the viral replication cycle (67). Furthermore, the ability to inhibit HIV-1 IIIB demonstrates that CTL can play an essential role in clearing infections by cytopathic viruses, a concept which has been questioned (28).

Although numerous studies have described the cytolytic activities of virus-specific CTL, there are relatively few data concerning the functional antiviral activity of these cells. At least two prior reports have addressed the ability of CTL to inhibit other viruses in vitro. Experiments with splenic lymphocytes from vaccinia virus-immune mice suggested that syngeneic CTL could lyse acutely infected cells in culture before the production of progeny virus (69). Another report illustrated the ability of virus-specific CTL clones to inhibit the production of lymphocytic choriomeningitis virus by an acutely infected macrophage cell line in culture (2). In our study, we extend these results by demonstrating that HIV-1-specific CTL are potent inhibitors of HIV-1 replication which mediate viral suppression by lytic and nonlytic mechanisms and are capable of completely clearing infectious virus from susceptible cell cultures.

Our studies address a number of controversies concerning HIV-1 suppression by $CD8^+$ cells. The data indicate that HIV-1-specific CTL are able to exert this antiviral activity, which many investigators have presumed to be noncytolytic (reviewed in reference 38). Activated CTL produce soluble factors which act without class I HLA restriction and do not require cell contact. Our results also extend previous studies on this subject by providing evidence that a trigger for the release of these factors is the recognition of viral antigen in the context of the appropriate restricting class I HLA molecule. Although these data do not exclude the existence of noncytolytic $CD8^+$ cells which produce soluble inhibitory factors, they suggest that CTL are sufficient to mediate the observed noncytolytic effects reported by others. Both CTL clones and polyclonal $CD8^+$ cells from infected individuals demonstrate HLA restriction in the experiments presented here, in which some target cells differ only in the expression of the relevant HLA molecule. The lack of HLA restriction observed by others may be a result of nonspecific stimulation of inhibitory factor production due to alloreactivity in mixed bulk cell populations, stimulation of $CD8^+$ cells with phytohemagglutinin, or CD3 cross-linking. Furthermore, we demonstrate that individual HIV-1-specific CTL clones at concentrations similar to those found in vivo are sufficient to suppress viral replication. Of note, many infected individuals may have CTL responses directed against multiple epitopes (reviewed in reference 24). In the present study, even CTL of a single specificity are highly inhibitory for HIV-1 replication.

HIV-1-suppressive soluble factors have recently received considerable attention. The elucidation of chemokine receptors which are necessary coreceptors for viral entry (1, 11, 13, 14) has revealed the potential role of various chemokines in blocking cellular infection (4, 9, 44). Our data indicate that

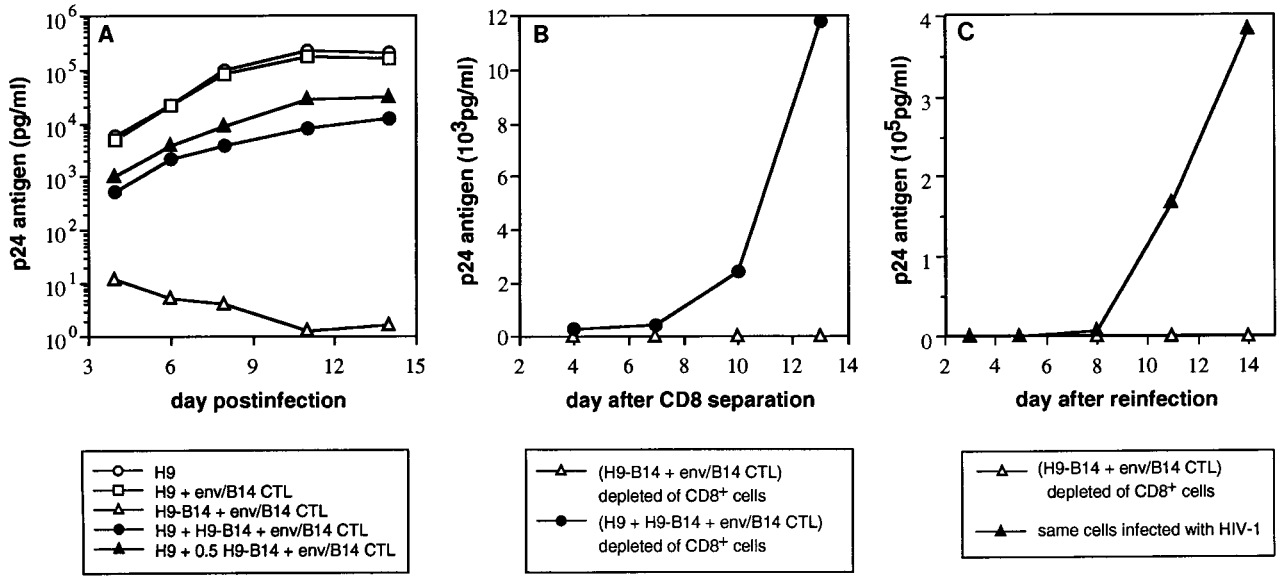


FIG. 6. Comparison of lytic and soluble components of HIV-1 suppression by CTL. (A) A mixture of HLA-matched and -mismatched infected cells was added to a CTL clone to compare inhibition by CTL with direct contact (HLA matched) or soluble factors (HLA mismatched). The HLA-matched infected cells served to trigger CTL to produce soluble factors acting on HLA-mismatched infected cells. H9 and H9-B14 cells acutely infected with HIV-1 IIIB were cocultured alone or mixed with the CTL clone Env/B14. (B) HIV-1 replication after removal of CTL from the cocultures above was determined by removal of CD8 cells from the wells containing (i) H9 and H9-B14 with Env/B14 and (ii) H9-B14 with Env/B14. After $CD8^+$ cell depletion of the cultures, the remaining cells were monitored as before for viral replication. (C) The infectability of the remaining culture-negative cells was assessed by rechallenge with HIV-1 IIIB under the same conditions as the initial infection.

HIV-1-specific CTL produce such soluble factors after antigen-specific activation, which can then act without cell contact or HLA restriction. CTL-derived inhibitory factors include MIP-1α, MIP-1β, RANTES, and other as yet undefined factors which are active on both M-tropic and T-tropic strains of HIV-1. Further investigation is required to determine the contribution of other proposed inhibitory factors such as stromal cell-derived factor (SDF-1) (4, 44), IL-16 (3), and $CD8^+$ cell-derived antiviral factor (CAF) (38). We have demonstrated previously that HIV-1-specific CTL clones produce other cytokines upon encountering their target cells (23), such as gamma interferon and tumor necrosis factor alpha/beta, which have been shown not to account for the antiviral effects of $CD8^+$ cells (38). Under conditions where CTL in direct contact with HLA-matched infected can clear detectable virus, bystander HLA-mismatched cells are only partially suppressed, suggesting another, more potent mechanism of viral inhibition by CTL.

Our data indicate a key role for cytolysis in the antiviral activity of CTL. The experiment in which infected H9 and H9-B14 cells were mixed in the presence of a B14-restricted CTL clone showed that bystander HLA-mismatched infected cells were moderately inhibited (10- to 100-fold) by soluble factors whereas HLA-matched infected cells under the same conditions were inhibited by at least $10^5$-fold (no detectable virus). Others have previously noted that cell contact is necessary for optimal inhibitory activity by bulk CD8 cells, also suggesting a possible role for cytolysis (59). We previously demonstrated that HIV-1-specific CTL clones appear to lyse acutely infected cells before the production of progeny virions and that Gag- and Env-specific CTL clones were more efficient than RT-specific clones (67). Here we present data which further support that finding. The inhibitory activity of the same CTL clones correlates with this lytic activity: RT-specific clones appear to be less efficient inhibitors of viral replication. In addition, CTL are able to act under conditions of maximal viral input, a phenomenon not described for noncytolytic $CD8^+$ cell-mediated HIV-1 suppression. Furthermore, CTL are capable of completely clearing detectable infectious virus in vitro, in contrast to other studies, which have failed to demonstrate irreversible inhibition of viral replication by bulk CD8 cells from infected individuals (61, 62). Potential explanations for this discrepancy include differences in experimental systems, such as our use of immortalized clonal target cell lines. Other investigators have used bulk PBMC $CD8^+$ cell depletion/repletion experiments to address this issue, and only infected individuals from whom viral replication was detectable after $CD8^+$ cell depletion were selected for study. Individuals with no recoverable virus after depletion, and therefore good in vivo control of viral replication, were excluded (61). Although isolating the cytolytic and noncytolytic effects of CTL on HIV-1 replication would provide the clearest comparison of these mechanisms, technical limitations preclude such an experiment at this time.

Despite the potency of viral suppression by HIV-1-specific CTL in vitro, the cytotoxic response appears to be incapable of clearing viral replication in infected individuals. Our data demonstrate clearance of infectious virus in cultures of $CD4^+$ lymphocytes, but it is possible that other cell types such as dendritic cells, follicular dendritic cells, and monocyte/macrophages represent resistant reservoirs. The inaccessibility of immunologically privileged sites to CTL may also impede clearance in vivo (17), and immunologic defects in $CD4^+$ cell help may play a role as well. These studies were performed with excess IL-2, which could be limiting in vivo (34). Other factors may include viral sequence variation leading to nonrecognition (10, 25, 47, 55) or antagonism (32), surface molecule alteration of infected cells (22, 30, 53, 54), direct viral immunosuppressive effects (12), and clonal exhaustion of CTL with disease progression (42). Further study is required to address these issues.

In summary, HIV-1-specific CTL exert vigorous suppressive activity on viral replication, which is initiated by an antigen-

specific interaction with infected target cells and executed by target cell lysis and soluble inhibitory factor-mediated pathways. CTL appear sufficient to mediate the observed antiviral effect of bulk $CD8^+$ cells from infected individuals. In view of the potency of viral suppression in vitro, it is important to elucidate mechanisms of viral escape from CTL in vivo. Understanding these processes will be important in developing preventative and therapeutic interventions for HIV-1 infection.

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