Inactivation of Human Immunodeficiency Virus (HIV)-1 Envelope-specific CD8+ Cytotoxic T Lymphocytes by Free Antigenic Peptide: A Self-Veto Mechanism?

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Summary

Free peptide has been found to inhibit cytotoxic T lymphocyte (CTL) activity, and veto cells bearing peptide–major histocompatibility complex (MHC) complexes have been found to inactivate CTL, but the two phenomena have not been connected. Here we show that a common mechanism may apply to both. CD8+ CTL lines or clones specific for a determinant of the human immunodeficiency virus (HIV) 1 IIIB envelope protein gp160, P18IIIB, are inhibited by as little as 10 min exposure to the minimal 10-mer peptide, I-10, within P18IIIB, free in solution, in contrast to peptide already bound to antigen-presenting cells (APC), which does not inhibit. Several lines of evidence suggest that the peptide must be processed and presented by H-2D^d on the CTL itself to the specific T cell receptor (TCR) to be inhibitory. The inhibition was not killing, in that CTL did not kill ^{51}Cr-labeled sister CTL in the presence of free peptide, and in mixing experiments with CTL lines of different specificities restricted by the same MHC molecule, D^d, the presence of free peptide recognized by one CTL line did not inhibit the activity of the other CTL line that could present the peptide. Also, partial recovery of activity could be elicited by restimulation with cell-bound peptide, supporting the conclusion that neither fratricide nor suicide (apoptosis) was involved. The classic veto phenomenon was ruled out by failure of peptide-bearing CTL to inactivate others. Using pairs of CTL lines of differing specificity but similar MHC restriction, each pulsed with the peptide for which the other is specific, we showed that the minimal requirement is simultaneous engagement of the TCR and class I MHC molecules of the same cell. This could occur in single cells or pairs of cells presenting peptide to each other. Thus, mechanistically the inhibition is analogous to veto, and might be called self-veto. As a clue to a possible mechanism, we found that free I-10 peptide induced apparent downregulation of expression of specific TCR as well as interleukin 2 receptor, CD69, lymphocyte function–associated antigen 1, and CD8. This self-veto effect also has implications for in vivo immunization and mechanisms of viral escape from CTL immunity.

The TCR of CD8+ class I MHC molecule–restricted cytotoxic T lymphocytes (CTL) binds a processed antigenic peptide, usually composed of 8–10 amino acids, fitted within the groove of a class I MHC molecule situated on the cell surface (1). Although peptide added free in solution may bind to the class I MHC molecule of the effector CTL, lysis of peptide-coated CTL by other CTL has been reported unlikely (2–4), probably because CTL are resistant to their own cytolytic mediators. Nevertheless, we found that a free peptide of optimal size (I-10: RGPGRAFVTI from HIV-1 gp160 envelope glycoprotein) (5, 6) almost completely blocked the specific CTL activity toward targets presensitized with the same peptide when added in solution during the 4–6 h ^{51}Cr release assay.

Several recent papers have described similar inhibitory phenomena by free antigenic peptide (7–11), but the mechanism of this inhibition is still controversial. Some papers suggest self-destruction (suicide) (8, 9, 12), some argue CTL–CTL killing (fratricide) (13), and some indicate a pronounced but transient inhibition or inactivation (anergy) (14). Also, as Su et al. (13) point out, the requirements for killing may be different from those for anergy. A possibly related phenomenon was seen earlier for CD4+ MHC class II–restricted Th cells, in which exposure of T cell clones to high concentrations of peptide for >6 h led to an anergic state lasting at least 7 d, although the cells were not killed because they could still respond to IL-2 (15–17). In this case, peptide had to be presented on class II

MHC molecules, but did not require APC other than the human class II MHC–positive T cells themselves. However, subsequent studies showed that such anergy induced by antigen presented on T cells was not due to lack of costimulation (18), and so the mechanism was distinct from that of anergy induced by antigen–MHC complexes in the absence of costimulation (19, 20).

Another situation in which CD8$^+$ CTL are inactivated but not always killed is termed the veto phenomenon (11, 21–24). The veto cell is a cell expressing the peptide–MHC complex recognized by the receptor of the CTL, which inactivates the CTL that targets it. Another CD8$^+$ cell is the most effective type of veto cell, but the veto phenomenon does not require engagement of the antigen-specific TCR of the veto cell, only its class I MHC molecules. Recent studies have shown that the CD8 molecule of the veto cell plays a role by binding the α3 domain of the MHC class I molecule of the CTL being vetoed (24). This phenomenon has been studied largely in cases of CTL specific for histocompatibility antigens (22), but recently cells presenting defined peptides have been shown to veto (11). However, in contrast to the studies of free peptide inhibition of CTL, CTL clones are resistant to the veto phenomenon (23). Thus, no connection has been made previously between the two phenomena.

In this study, we show that the inhibition of CTL is initiated by the binding of antigenic peptide for which the CTL is specific to the MHC molecules on the CTL's own surface, and is not caused by either the peptide presented only on other cells including sister CTL, or by direct interaction of the TCR with free peptide. Using pairs of CTL lines of differing specificity but similar MHC restriction, each pulsed with the peptide for which the other is specific, we show that the minimal requirement is simultaneous engagement of the TCR and class I MHC molecules of the same cell. The transient anergy is associated with downregulation of the TCR and several accessory cell surface molecules.

This mechanism of inhibition characterized here is reminiscent of the veto mechanism. However, it is not simply the classic veto phenomenon, because CTL presenting the peptide to other CTL do not inhibit them. We suggest that indeed the inhibition by free peptide is analogous at the molecular level to the veto phenomenon, but that CTL clones require a stronger signal through their MHC class I molecules, using a TCR rather than simply CD8 interaction with the α3 domain. Thus, free peptide inhibition of CTL clones may be considered a process of "self-veto." This self-veto phenomenon may be important not only for in vitro studies, but also in vivo for its possible effect on viral persistence and CTL inactivation by viral products.

Materials and Methods

Animals. BALB/c (D^d, L^d, K^d) mice were obtained from Charles River Japan Inc. (Tokyo, Japan) and B10.A (D^d, L^d, K^k) from Sankyo Laboratory Inc. (Tokyo, Japan). Mice were used at 6–12 wk of age.

CTL Lines and Clones. The CTL line (LINE-IIIB) and clones specific for the HIV-1 envelope protein of the IIIB isolate (gp160IIIB) were generated as described (25). Both the gp160IIIB-specific CTL line and the clones were restricted by the D^d class I MHC molecule and were specific for an immunodominant epitope P18IIIB (RIQRGPGRAFVTIGK). The minimal active peptide was shown to be the 10-mer, P18-I-10 (5, 6, 26). The CTL line (LINE-MN) specific for the HIV-1 envelope of the MN isolate (gp160MN) was obtained from vMN (gp160MN-expressing recombinant vaccinia virus)–immune BALB/c spleen cells (27). It was stimulated with P18MN (RIHIGPGRAFYT-TKN)-pulsed irradiated syngeneic spleen cells and with rat ConA supernatant added on the second day and maintained by biweekly repetitive stimulation.

Synthetic Peptides. The peptides are named according to the last amino acid residue and the length, except for the original P18IIIB and P18MN peptides. Peptides were synthesized and purified as described previously (25–27).

mAbs. The following mAbs were used: anti-CD4 (RL172.4) (28) for depleting P18IIIB-specific I-A^d–restricted (29) CD4$^+$ Th cell line (HT-4) (6) and anti-K^k (30) for depleting B10.A spleen cells with rabbit C (Cederlane Laboratories Ltd., Hornby, Ontario, Canada); FITC-labeled anti-CD3 (2C11 [31]), anti-IL-2Rα (32), anti-CD69 (33), or anti-Vβ-8.1 (34), anti-D^d (α3 domain) (34-2-12 [35]), anti-L^d (30-5-7 [36]), and anti-K^d (31-3-4 [36]) for cell surface staining.

Inhibition of Serum Activity with Angiotensin-converting Enzyme (ACE) Inhibitor. The ACE (EC3.4.15.1 peptidyl/dipeptide hydrolase)-specific inhibitor captopril (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 100 μM and added to the culture at 10^{-5} M 30 min before mixing with the inhibitory peptide.
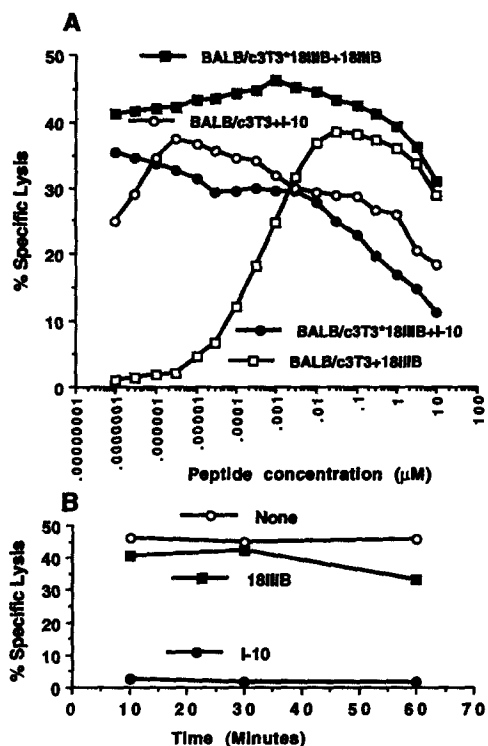
CTL Assay. Cytotoxicity was assessed in a standard 5-h ^{51}Cr release assay as described previously (37), with ^{51}Cr-labeled targets, as indicated in the figure legends. SEM of triplicate cultures was always <5% (and often <3%) of the mean.

FACS® Analysis. For direct one-color staining to determine the effect of free peptide treatment of the gp160IIIB-specific CTL lines or clones on their surface molecule expression, 10^6 cells were incubated at 37°C for 1 h with free I-10 and then washed three times with RPMI 1640 to remove free peptide. Then, 1 μg of each indicated FITC-labeled antibody was added to the cell pellet for a 40-min incubation at 4°C. All reagents were pretitered and used in amounts known to be saturating on positive controls. The cells were washed three times and resuspended at 10^6 cells/0.5 ml in PBS/BSA/azide for analysis by FACScan® analyzer (Becton Dickinson Immunochemistry Systems, Mountain View, CA). Dead and damaged cells were excluded from the analysis by propidium iodide gating.

Results

Free Peptides Inhibit CTL Activities in a Dose-dependent Manner. The minimal epitope of CD8$^+$ CTL lines or clones specific for an immunodominant determinant of the HIV-1 IIIB envelope protein gp160, 18IIIB (315–329) (RIQRGPGRAFVTIGK), presented by the murine class I MHC molecule H-2D^d, has been identified as a 10-mer peptide, I-10 (RGPGRAFVTI) (5, 6, 26). We have noticed that we could not show a clear concentration dependence when the minimal size free I-10 was added together with ^{51}Cr-labeled fibroblast targets during the 4-h assay, al-

though the longer peptide, 18IIIB, produced a normal titration curve (Fig. 1 A). As a possible explanation, when we used peptide-pulsed targets, we observed that free I-10 strongly inhibited CTL activity even at very low concentrations, whereas the longer peptide, 18IIIB, inhibited the activity only at high concentrations (Fig. 1 A). Similarly, high concentrations of free 18IIIB showed some inhibition when used with the unpulsed fibroblast targets. Similar results were obtained in five other experiments, including one with I-10–pulsed targets. Thus, inhibition by free I-10 superimposed on sensitization of targets by I-10 led to a relatively flat net dose–response curve over a wide range, with less net killing than was seen with the longer peptide. Free peptide inhibition could also be observed in another system, using a different epitope (AH2-I9: residues 39–47 from the HIV-1 reverse transcriptase restricted by the K^k MHC molecule [data not shown]). These results suggested that free epitopic peptides from the virus may inhibit spe-
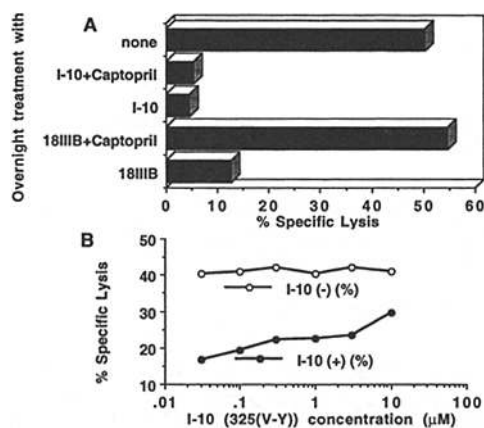
cific CTL activity against virus-infected targets, and this may be another mechanism of virus-specific CTL inactivation in vivo. A number of the experiments detailed below were carried out with both the HIV reverse transcriptase and envelope peptides with similar results, but for simplicity only the envelope peptide experiments are shown.

Titration and Kinetics of I-10 Treatment for CTL Inhibition. To further investigate the mechanism of the inhibition, we pretreated the CTL line or clones with various concentrations of I-10 for 1 h in the absence of targets, and then added these cells to the assay culture after complete removal of the free peptide. Profound reduction of CTL activity was observed when I-10–specific CTL lines or clones were cocultured briefly with the free minimal peptide I-10 at >1 μM, and half-maximal inhibition was achieved at between 0.01 and 0.1 μM peptide for only 1 h (data not shown). Surprisingly, <10-min pretreatment with 1 μM of I-10 appeared to be sufficient to induce inhibition (Fig. 1 B). Shorter times could not be investigated because of the time required for centrifugation and washing. In contrast, although 1 μM of the 15-mer peptide 18IIIB showed some inhibitory activity with 1-h pretreatment (Fig. 1 B), >2 h of treatment was necessary to generate strong inhibition (Fig. 2 A and data not shown).

Requirement for Peptide Processing. The inhibition could not be observed when I-10 was presented to the CTL in an already cell-associated form, either I-10–pulsed BALB/c.3T3 fibroblast (Neo*I-10) or endogenously synthesized in a gp160-transfected BALB/c.3T3 fibroblast (15–12) (data not shown and see below). These results suggested that some processing steps are required for 18IIIB to be inhibi-

Figure 1. (A) Free epitopic peptides inhibit CTL activity in a dose-dependent manner. Either 5 × 10³ untreated ⁵¹Cr-labeled BALB/c.3T3 fibroblast targets (O–O, □–□) or an equal number of P18IIIB-prepulsed ⁵¹Cr-labeled BALB/c.3T3 target cells (●–●, ■–■) were incubated with 5 × 10⁴ cells of a P18IIIB-specific CTL line (LINE-IIIB) in the presence of various amounts of free antigenic peptides (P18IIIB [□–□, ■–■] or I-10 [O–O, ●–●]) in 96-well round-bottom microtiter plates during the 4 h assay. (B) Kinetics of I-10 treatment for CTL inhibition. 10⁶/ml of LINE-IIIB cells were incubated with 1 μM I-10 for various times at 37°C in complete T-cell medium (CTM)[1] (25) and washed three times. Then 5 × 10⁴ treated LINE-IIIB cells were added to 5 × 10³ of ⁵¹Cr-labeled P18IIIB-prepulsed targets for 4 h. SEM of triplicate cultures was always <5% of the mean.

Figure 2. CTL inhibition by free peptide requires processing and presentation of peptide by class I MHC molecules. (A) Captopril abrogates CTL inhibition by P18IIIB. 10⁶/ml LINE-IIIB were incubated with 10⁻⁵ M captopril, a dipeptidase inhibitor that inhibits processing of P18IIIB to I-10 (26), together with 1 μM P18IIIB or I-10 overnight. Then the CTL were washed three times, and 5 × 10⁴ treated LINE-IIIB cells were added to 5 × 10³ ⁵¹Cr-labeled P18IIIB-prepulsed targets for 4 h. (B) Effect of coculturing with competitor peptide during 4-h CTL assay. 5 × 10³ P18IIIB-prepulsed ⁵¹Cr-labeled BALB/c.3T3 fibroblast targets were incubated with 5 × 10⁴ P18IIIB-specific LINE-IIIB cells at the indicated concentrations of competitor peptide together with (●–●) or without (O–O) 0.1 μM I-10 during the 4-h assay. SEM of triplicate cultures was always <5% of the mean.

tory, in contrast to I-10, which may bind directly to the surface MHC molecules of the CTL. To confirm this interpretation, we added the dipeptidase inhibitor captopril, which inhibits serum processing of 18IIIB to I-10 (26), together with either 18IIIB or I-10 in an overnight culture with the CTL line, and found that captopril could abrogate the inhibitory activity of 18IIIB but not I-10 (Fig. 2 A). These findings were reproducible in two additional experiments. Also, since captopril did not affect I-10 inhibition of CTL activity, it should not be acting at other steps, such as peptide binding to MHC. In addition, inhibition by 18IIIB, but not by I-10, requires the presence of FCS (data not shown). Thus, proteolysis of the 15-mer peptide is necessary for it to inhibit. Also consistent with presentation of processed peptide by D^d as a requirement for inhibition, we found that treatment of CTL with mAbs specific for D^d partially prevented the inhibition (data not shown).

CTL Inhibition Can Be Partially Abrogated by Competitive Peptide. To test whether inhibition required binding of the free I-10 to the TCR on the CTL and not just to the MHC molecule, we synthesized I-10 (325(V-Y)) with a single substitution at position 325, which we have identified as the major site for interacting with the TCR (25, 27). We have previously demonstrated that HIV-1 IIIB–specific CTL tend to see aliphatic amino acids at position 325, whereas MN-specific CTL see aromatic or cyclic amino acids at this position (38). This substituted peptide I-10 (325(V-Y)) could not be recognized by IIIB-specific CTL at all, although it binds to D^d because it can be seen by MN-specific CTL with D^d (data not shown). Also, studies with sequentially added peptides indicated that the substituted peptide was not an antagonist (39–41) (data not shown). Thus, the peptide cannot interact with the TCR of IIIB-specific CTL despite its binding to the D^d class I MHC molecule. As shown in Fig. 2 B, peptide I-10 (325(V-Y)) did not itself inhibit, but competitively blocked the inhibition induced by I-10 in a dose-dependent manner during the 4-h CTL assay (Fig. 2 B). Thus, peptide must bind to both the MHC molecule and the TCR on the CTL to inhibit. Also, since the modified peptide cannot compete for binding to the TCR, this result also confirms that inhibition is mediated by peptide–MHC complexes and not direct binding of the peptide to the TCR.

I-10–pulsed T Cells Did Not Inhibit CTL Activity. Since free peptide had to bind to the appropriate class I MHC molecule and be presented to the TCR of the CTL being inhibited, we asked why peptide already bound to D^d on fibroblasts did not inhibit. Perhaps the peptide had to be presented on another type of cell. Therefore, we pulsed I-10 onto a whole–spleen cell population, chosen to have the same D^d molecule but a different H-2K molecule so that the cells could be depleted afterwards. We did not observe any inhibition when the CTL were cocultured for 1 h with irradiated B10.A (D^d and K^k) spleen cells prepulsed with I-10 and then treated with anti-K^k mAb and rabbit C to remove B10.A cells (Fig. 3 A). Some apparent cold-target inhibition by the peptide-pulsed B10.A cells is eliminated when these are removed. These results
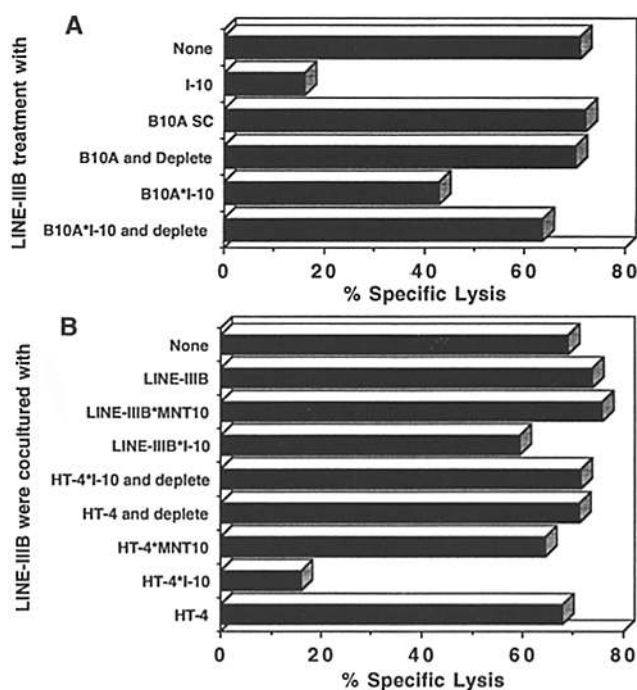
Figure 3. Inability of spleen cells and other T cells to mediate peptide inhibition of CTL lines. (A) Effect of I-10–pulsed B10.A spleen cells on LINE-IIIB inhibition. 5 × 10⁶/ml B10.A (D^d and K^k) spleen cells (APC) were incubated with 1 μM I-10 for 1 h in CTM (25), 3,300 rad irradiated, washed three times, and mixed with 5 × 10⁴ IIIB-specific CTL line cells for another 1 h. The mixed cells were then treated with anti-K^k mAb and rabbit C to deplete B10.A cells and cocultured with 5 × 10³ ⁵¹Cr-labeled gp160-expressing BALB/c3T3 fibroblast (15-12) targets for 4 h. (B) Effect of coculturing with I-10–pulsed T cells on CTL inhibition. 10⁶/ml LINE-IIIB were cocultured with an equal number of CD8⁺ LINE-IIIB prepulsed for 1 h with 1 μM I-10 or MNT10, or with the P18IIIB-specific CD4⁺ helper T cell line, HT-4, prepulsed with 1 μM I-10 for 1 h. Where indicated, the mixed cells were then treated with anti-CD4 mAb (RL174) and rabbit C to deplete the CD4⁺ HT-4 line. Then the effector cells were cocultured with 5 × 10³ ⁵¹Cr-labeled P18IIIB-prepulsed targets for 4 h. SEM of triplicate cultures was always <5% of the mean.

were reproducible in three experiments. These data strongly indicated that the free peptide I-10 does not work via binding to APC contamination in the CTL line, but only by binding to the CTL themselves. These data also argue against inhibition by veto cells in the spleen, which should inhibit the CTL to which they present peptide. Failure to see such a veto phenomenon is consistent with the resistance of CTL clones to veto (23).

If the free peptide requires processing so that it can bind to MHC molecules, but does not act when bound to other cells, it may have to be presented by T cells to inhibit. Therefore, we tested the effect of presention by other CD4⁺ or CD8⁺ T cells. Taking advantage of the D^d-expressing CD4⁺ helper T cell line, HT-4 (6), I-10–specific CTL line (LINE-IIIB) cells were mixed with an equal number of I-10–pulsed HT-4 or an equal number of I-10–pulsed LINE-IIIB cells before being added to the ⁵¹Cr-labeled Neo*I-10 targets for the 4-h assay culture. In contrast to unpulsed HT-4, I-10–pulsed HT-4 significantly inhibited the activity of LINE-IIIB, whereas I-10–pulsed CD8⁺

CTL of the same LINE-IIIB did not (Fig. 3 *B*). We have found that LINE-IIIB did efficiently kill ^{51}Cr-labeled HT-4 cells when pulsed with I-10, whereas they did not lyse ^{51}Cr-labeled I-10–pulsed LINE-IIIB as targets (data not shown). Thus, we speculated that I-10–pulsed HT-4 probably acted as cold target inhibitors in the assay culture. Therefore, we depleted the culture of I-10–pulsed HT-4 cells by treatment with rat anti–mouse CD4 mAb (RL174) plus rabbit C after a 2-h incubation with LINE-IIIB, and then added the residual LINE-IIIB to the assay system for an additional 4 h. Depletion of I-10–pulsed HT-4 cells completely abrogated the inhibition. This result, reproduced in two additional experiments, suggests that the peptide, I-10, does not inhibit specific CD8^{+} CTL when presented by their fellow CD8^{+} T cells, and the inhibition by I-10–pulsed CD4^{+} T cells appears to be by a different mechanism, cold-target inhibition, which does not explain the effect of free peptide on CTL. This result also excludes a classic veto mechanism, in which CD8^{+} cells presenting peptide to the TCR of a CTL inhibit it (11, 21–23).

Fratricide May Not Be the Cause of Inhibition. These results demonstrate that free antigenic peptide must bind to the MHC molecule on the surface of effector CTL to downregulate their cytolytic activity. To distinguish whether the mechanism of inhibition was CTL fratricide, suicide, or anergy induced when the TCR interacts with a peptide–MHC complex on the surface of the CTL itself, we ^{51}Cr labeled some of the same CTL line as targets and found that they did not kill each other in the presence of free peptide (data not shown). Moreover, in mixing experiments with two non–cross-reactive CD8^{+} CTL lines (LINE-IIIB and LINE-MN) specific for two homologous peptides, 18IIIB (or I-10) and 18MN (or MNT10) from different HIV-1 isolates, both presented by the same MHC molecule, H-2D[d], we found that the presence of free peptide recognized by one CTL line but able to bind to H-2D[d] on both CTL lines did not inhibit the cytolytic activity of the other syngeneic CTL line for its targets, as would be expected if the mechanism had been fratricide (Fig. 4, reproduced in three additional experiments). Furthermore, MNT10-prepulsed LINE-IIIB was not inhibited at all when cocultured with LINE-MN and, conversely, I-10–prepulsed LINE-MN was not inhibited when cocultured with LINE-IIIB (see Fig. 5). Thus, the mechanism of CTL inhibition by the free epitopic peptide appears not to be fratricide, inhibition by one T cell of another T cell presenting the specific peptide, or the release of inhibitory cytokines. Rather, it appears that the peptide must be presented on the T cell's own MHC molecules, to the cell's own TCR specific for that peptide–MHC complex.
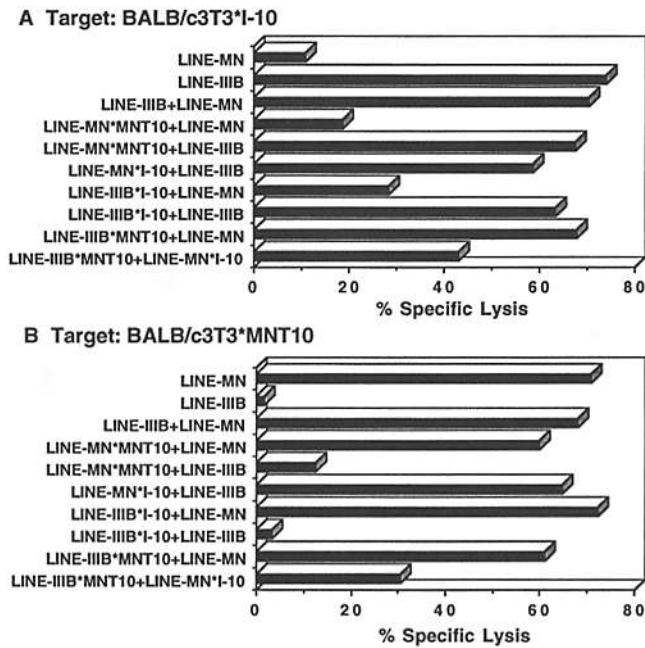
Dual Engagement Requirement. The possibility remained that simultaneous occupancy of both the TCR and MHC molecule on the CTL was all that was required, so that two CTL lines specific for different peptides on the same MHC molecule could inhibit each other if each was pulsed with the peptide for which the other CTL was specific and they were washed and mixed. This experiment differs from the previous one in that the CTL presenting one peptide in its

A IIIB-specific inhibition

B MN-specific inhibition
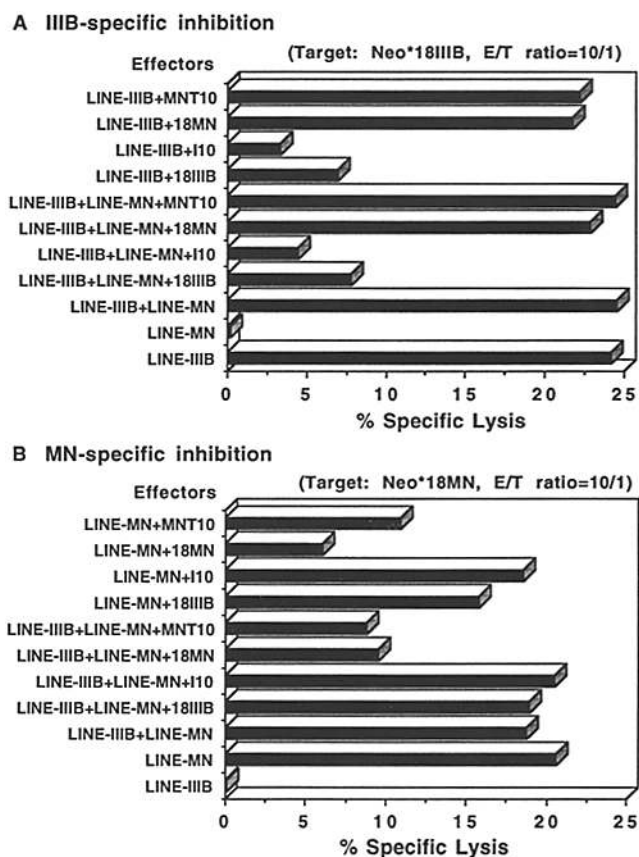
Figure 4. Fratricide is not the cause of inhibition by free peptide. 5 × 10^{4} treated cells of LINE-IIIB and/or LINE-MN were added to 5 × 10^{3} ^{51}Cr-labeled P18IIIB-prepulsed targets (*A*) or P18MN-prepulsed targets (*B*) in the presence of either 1 μM of free P18IIIB (I-10) or an equal amount of free P18MN (MNT10) for 4 h. SEM of triplicate cultures was always <5% of the mean.
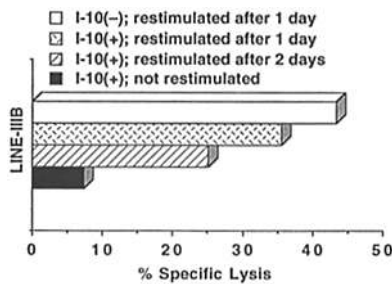
MHC molecule also can engage its TCR with the peptide–MHC on the other T cell at the same time. To test this possibility, we mixed LINE-MN pulsed with I-10 and LINE-IIIB pulsed with MNT10 and found that both were inhibited (Fig. 5), tested on their respective targets. Only this configuration of pulsed cells showed inhibition. This inhibition in *trans* appears less efficient than in *cis*, that is, when the TCR was engaging the peptide–MHC complex on the same cell, but was reproducible and statistically significant. In eight inhibition experiments in four independent studies similar to the one shown in Fig. 5, the mean percentage of inhibition was 40.3 ± 2.69% (*P* <0.001 by Student's *t* test). Thus, the mechanism of inhibition appears to require that both the MHC molecules and the TCRs of the CTL be engaged simultaneously ("dual engagement"), either on the same cell or in a conjugate between two or more cells. This dual engagement mechanism is reminiscent of, but distinct from, the veto process (see Discussion).

I-10–treated CTL Activity Could Be Partially Restored by Restimulation. If the mechanism of inhibition were apoptosis of CTL, cytotoxic activity should not be recovered by restimulation, whereas if it were anergy, activity might be recoverable. Both the downregulated CTL line (Fig. 6) and
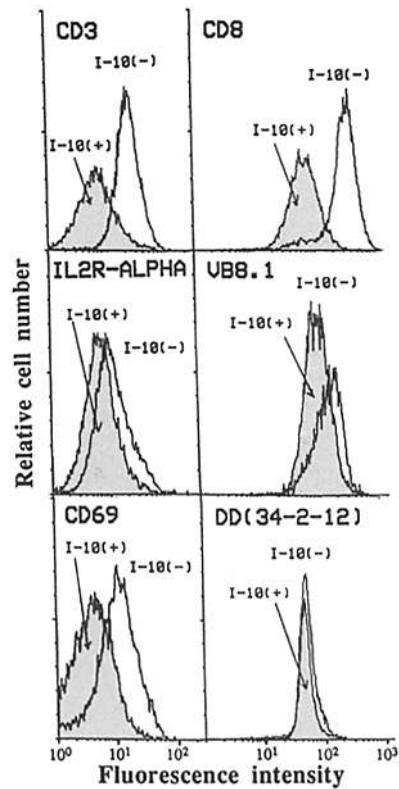
A Target: BALB/c3T3*I-10

Bars labeled (top to bottom):
LINE-MN
LINE-IIIB
LINE-IIIB+LINE-MN
LINE-MN*MNT10+LINE-MN
LINE-MN*MNT10+LINE-IIIB
LINE-MN*I-10+LINE-IIIB
LINE-IIIB*I-10+LINE-MN
LINE-IIIB*I-10+LINE-IIIB
LINE-IIIB*MNT10+LINE-MN
LINE-IIIB*MNT10+LINE-MN*I-10

X-axis: % Specific Lysis (0, 20, 40, 60, 80)

B Target: BALB/c3T3*MNT10

Bars labeled (top to bottom):
LINE-MN
LINE-IIIB
LINE-IIIB+LINE-MN
LINE-MN*MNT10+LINE-MN
LINE-MN*MNT10+LINE-IIIB
LINE-MN*I-10+LINE-IIIB
LINE-IIIB*I-10+LINE-MN
LINE-IIIB*I-10+LINE-IIIB
LINE-IIIB*MNT10+LINE-MN
LINE-IIIB*MNT10+LINE-MN*I-10

X-axis: % Specific Lysis (0, 20, 40, 60, 80)

Figure 5. Dual engagement may be the cause of inhibition by free peptide. 2×10^6/ml non–cross-reactive CTL lines, LINE-IIIB and LINE-MN (P18MN-specific CTL line), were pretreated with either 1 μM I-10 or MNT10 for 1 h at 37°C. After being washed three times, 5×10^4 treated cells of LINE-IIIB and/or LINE-MN were added to 5×10^3 ^{51}Cr-labeled I-10–prepulsed targets (A) or MNT10-prepulsed targets (B) for 4 h. SEM of triplicate cultures was always <5% of the mean.

clone RT-3 (not shown) treated with 1 μM I-10 for 1 h could be restored to almost 80% of their original activities by restimulation with I-10–expressing BALB/c fibroblasts even 2 d after the free peptide treatment (reproducible in three experiments). Even if the cells were pretreated with I-10 for a full 24 h and then restimulated with the gp160 transfectant 15-12, 83% of the activity could be recovered (data not shown). This result suggests that the major mechanism of inhibition is temporary self-inactivation (anergy) rather than apoptosis (suicide). We also could not detect any DNA ladders in the CTL treated with I-10 for 2 h and

Legend:
□ I-10(–); restimulated after 1 day
▨ I-10(+); restimulated after 1 day
▧ I-10(+); restimulated after 2 days
■ I-10(+); not restimulated

Y-axis: LINE-IIIB
X-axis: % Specific Lysis (0, 10, 20, 30, 40, 50)

Figure 6. CTL inhibited by free I-10 can be partially restored by restimulation with I-10–expressing cells. 4×10^6/ml LINE-IIIB were treated with 1 μM of I-10 for 1 h and washed three times. After 1 or 2 d further incubation at 10^6/ml, treated cells were restimulated with 10^5/ml mitomycin C–treated I-10–expressing syngeneic BALB/c 3T3 fibroblasts together with IL-2 for another 5 d in a 24-well culture plate, harvested, and tested for their cytolytic activities against 5×10^3 ^{51}Cr-labeled P18IIIB-prepulsed targets at an E/T ratio of 20:1. SEM of triplicate cultures was always <5% of the mean.

Panels labeled: CD3, CD8, IL2R-ALPHA, VB8.1, CD69, DD(34-2-12), each with I-10(+) and I-10(–) traces.
Y-axis: Relative cell number
X-axis: Fluorescence intensity (10^0, 10^1, 10^2, 10^1, 10^2, 10^3)

Figure 7. Downregulation of surface markers on the CTL treated with free I-10. 10^6 cells were incubated for 1 h with free I-10 and washed three times. Then 1 μg of the indicated FITC-labeled antibody was added to the cell pellet for a 40-min incubation at 4°C. The cells were washed three times and resuspended at 10^6 cells/0.5 ml in PBS/BSA/azide for analysis by FACScan®.

then cultured without peptide for an additional 24 h, or pulsed with I-10 for a full 24 h (data not shown).

Downregulation of Surface Markers on the CTL Treated with Free I-10. As a clue to a possible inhibitory mechanism, we found that free I-10 induced apparent downregulation of expression of specific TCR (Vβ8.1 on clone RT-2 or RT-3 [Takahashi, H., and Y. Nakagawa, unpublished observation]) as well as CD3, IL-2R (α, and β not shown), CD69 (activated T cell marker), LFA-1 (not shown), and CD8 (Fig. 7). However, class I MHC molecules such as D^d (Fig. 7), K^d, or L^d (data not shown) did not show any downmodulation on the I-10–treated CTL. The same pattern of downregulation was seen in five independent experiments, as well as one in which the cells were maintained in suspension (albeit to a slightly lesser extent). It was also observed after 24 h of exposure to I-10, and the expression remained partially downregulated 24 h after a 1-h exposure (data not shown). The lack of effect on class I MHC molecules suggests that the FACS® analysis results are not merely caused by downsizing of the cells or a generalized effect on all surface molecules. Thus, the inhibition of CTL activity is concurrent with a downmodulation of surface activation markers and specific TCR, the latter resembling that reported for $CD4^+$ class II MHC–restricted T cells exposed to high concentrations of specific peptide (17).

Discussion

In this study, we found that exposure of CD8^{+} CTL to a peptide corresponding to the minimal epitope, free in solution, leads to strong inhibition of the cytolytic activity of the epitope-specific CTL. Our data suggest that this effect requires dual engagement of TCR and MHC molecules on the same T cell and involves downregulation of TCR and several other surface molecules without cell death. Although shown to be distinct from the classic veto mechanism (21–24), which does not act on long-term CTL lines and clones in vitro, the dual engagement requirement is nevertheless rather analogous to the veto mechanism on a molecular level, so that the inhibition by free peptide may be considered a self-veto process, as discussed below.

Several papers have reported that free epitopic peptide can inhibit the specific activities of CD8^{+} CTL (7–11). However, the mechanism of the inhibition is still controversial, and previous studies have not examined a dual engagement requirement. Su et al. (13) concluded that the mechanism of inhibition by free cognate peptide is "fratricide" rather than "suicide" (8, 9, 12) based on experiments using CTL isolated with the microdrop separation technique using gel agarose. However, although no killing was observed in the isolated cells in microdrops, functional anergy could not be tested. Thus, there is no real discrepancy with our study, in which we observe anergy but not killing, and in which the process appears to be able to occur in single cells. LaSalle et al. (42) observed that anergy required cell contact when peptides presented by class II MHC molecules were used, but it is not clear that the mechanism is the same as described here for class I MHC presentation. If, as our data suggest, either a single cell or two-cell mechanism can occur, one or the other may predominate depending on cell density and peptide concentration. Thus, the differences among the studies may be explained, at least in part, by differences in these parameters. Also, as Su et al. (13) point out, the requirements for anergy may be different from those for cell death. For example, the study demonstrating fratricide was carried out at 10^7-fold higher peptide concentration than required for 50% lysis of targets, whereas the anergy induction occurred in a peptide concentration range similar to that required for target sensitization (Fig. 1 A). Thus, high dose inhibition may be playing a role in some studies and not others, invoking a mechanism different from that of free peptide at lower concentrations.

To investigate fratricide as a mechanism in our system, we used two distinct and non–cross-reactive CD8^{+} CTL lines, LINE-IIIB and LINE-MN, specific for the homologous peptides I-10 (18IIIB) or MNT10 (18MN), respectively, and restricted by the same class I MHC molecule, H-2D[d]. We did not detect any inhibition when the LINE-IIIB cells were prepulsed or mixed with soluble 18MN or MNT10 and cocultured with LINE-MN cells that should kill such peptide-pulsed LINE-IIIB cells if the mechanism were fratricide, and vice versa in the reciprocal combination. Moreover, when half the CTL line cells were pulsed

with the peptide for which they were specific and cocultured with the other half, the cytolytic activity of the unpulsed cells was not inhibited. These results exclude a classic veto mechanism (11, 21–24). In addition, we confirmed that ^{51}Cr-labeled I-10–pulsed CD8^{+} CTL are not killed by LINE-IIIB, consistent with resistance of CTL to lysis (2–4). We conclude that fratricide is not the mechanism of inhibition in our system.

Furthermore, we have shown that I-10–treated CTL can be restored to almost 80% of their original activity by restimulation 2 d after peptide treatment. This result and the absence of obvious DNA ladders in the treated CTL also strongly suggest that the principal mechanism is not suicide. Taken together, these results suggest that the major mechanism of inhibition, under our conditions, is transient self-inactivation (anergy or paralysis), not cell death. This interpretation is consistent with the downregulation of receptors we observed. However, it should be noted that the difference between cell death and anergy may depend on the state of the CTL in the conditions of the experiment, such as their bcl-2 levels, so that either outcome may be possible under different circumstances, even when the signaling mechanism is the same.

The effect of peptide length may be an important variable not analyzed in previous studies that may explain some differences in results. Here we show that longer peptides, such as 18IIIB, need processing by protease(s) present in FCS that can be inhibited by specific inhibitors such as captopril (26); thus, only the optimal-sized peptide, such as I-10, caused rapid and strong inhibition of CTL activity, whereas it takes >2 h for the 15-residue peptide 18IIIB to inhibit. Therefore, some discrepancies between studies may be due to use of longer peptides to analyze the inhibitory mechanism. For example, the difference between the FACS® analysis data of Robbins and McMichael (10) and ours may come from the length of the peptide used in the assay, in that they demonstrated downmodulation of CD8 but not of IL-2 receptor or TCR in the presence of free 13-mer peptide from influenza nucleoprotein, which required 10 h for inhibition and was more variable, as we have seen for the 15-mer 18IIIB.

To better understand the minimal signaling requirements for free peptide to inhibit, we also studied the efficacy of other APC in inducing the inhibition. First, we found that BALB/c.3T3 fibroblasts expressing I-10, either externally pulsed or internally synthesized, did not inhibit the activity of specific CD8^{+} CTL. Second, I-10–pulsed D[d]-expressing whole spleen cells from B10.A mice, containing a variety of APC, did not affect the CTL activity when the B10.A cells were removed before the ^{51}Cr-release assay to avoid cold-target blocking. (Since the spleen cells also contain T cells that could act as veto cells, these results also help to exclude a classic veto cell mechanism [11, 21–24].) Third, we found that presentation of I-10 by the BALB/c CD4^{+} Th cell line, HT-4, did not inhibit CD8^{+} CTL, except by cold-target inhibition, which could be eliminated by de-

pletion of the CD4$^+$ cells. In contrast, CTL line cells that could not be killed when pulsed with peptide also did not act as cold targets and did not inhibit. Time-dependent morphological changes in isolated CTL with free peptide (data not shown), similar to the morphological change observed by Walden and Eisen (8), if indicative of the same phenomenon, also exclude cold target inhibition by other CTL and suggest action at the single cell level. Thus, presentation of peptide by any other cell does not mimic the effect of free peptide.

Nevertheless, the data indicate that the free peptide does not act in the free state, but must be presented by a class I MHC molecule. Captopril abrogation of inhibition by 18IIIB but not I-10 suggests that the inhibition requires binding of the minimal peptide to the class I MHC molecule on the CD8$^+$ CTL. This conclusion is supported by partial blocking of inhibition by pretreatment of CTL with anti-D^d (data not shown) and by the fact that a noninhibitory, nonantagonistic I-10 variant with a single substitution at a key TCR-interacting site competed against the inhibitory activity of the unmodified I-10. The modified peptide could not compete for binding to the TCR because it had neither agonist nor antagonist activity despite binding to D^d. Thus, competition must be for binding to the MHC molecule, a further indication that free peptide has to bind to the MHC molecule to inhibit. Vitiello et al. (7) similarly concluded that the peptide must be presented on the MHC molecule of the CTL itself from experiments using D^b-restricted influenza nucleoprotein–specific CTL derived from chimeric mice in which the CTL were of different genetic origin not expressing D^b. However, the fact that CTL–CTL presentation of peptide did not produce cold-target inhibition, and the inability of this mechanism to explain the downregulation of multiple surface molecules on the CTL, make cold-target inhibition of a single CTL by its own MHC molecules presenting peptide (7) unlikely. Taken together, these results, which demonstrate a requirement for binding to the class I MHC molecule but exclude presentation on other cells, indicate that the free peptide must be presented on the CTL themselves to induce specific CD8$^+$ CTL inhibition.

How then does presentation of the peptide on the CTL's own MHC molecules differ from presentation on the same MHC molecule of another cell? The experiments in which CTL of different specificity but similar MHC restriction, pulsed with each other's peptide, can inhibit each other (Fig. 5), in contrast to the case in which the peptide for only one of the CTL is present (Fig. 4), show that the minimal requirement for inhibition is simultaneous occupancy of the TCR and MHC molecule on the CTL. This double-pulsing experiment of Fig. 5 creates a situation in which the CTL cannot see the peptide on their own MHC molecules, but must see it on another CTL, and yet each CTL has both its MHC molecules and TCR engaged (Fig. 8 B). It allows us to conclude that such simultaneous dual engagement of MHC and TCR on the same cell is required. In the normal circumstance with a single CTL line and a single peptide, this situation can occur between pairs

of the same CTL at high density incubated with free peptide (Fig. 8 C), as well as on isolated single cells (Fig. 8 A). When cells of the same clone are not together at high density, as may often be the case in nature, the single-cell mechanism may be the only one available. Presentation in the same cell is possible because the cell surface is not smoothly convex, but has many projections and invaginations, and independent evidence for such a functional interaction has been obtained (Koenig, S., personal communication). If a mixture of peptides and CTL specific for them occurs, as during lysis of virally infected cells, the situation in Fig. 8 B, as created in the experiment shown in Fig. 5, may also arise. Thus, our results suggest that the most likely mechanism of this inhibition may be signal transduction within the CTL by having its TCR ligated to its own MHC molecules in cis or in a reciprocal interaction with a sister cell's TCR and MHC molecules in trans, resulting in reversible downregulation of surface molecules.
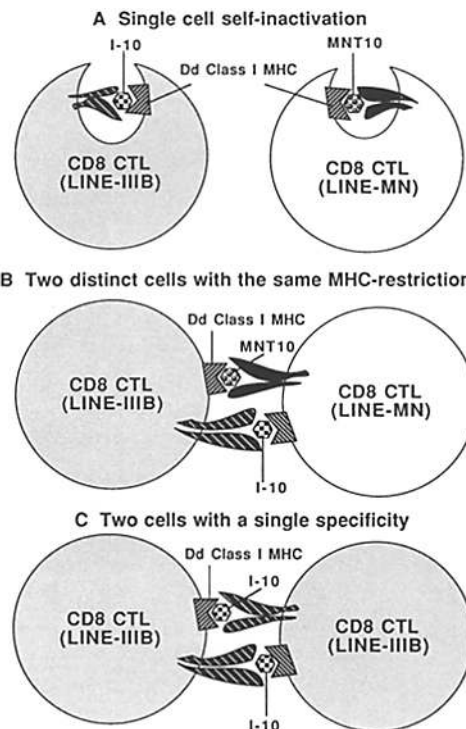
Figure 8. Model of the self-veto or dual engagement mechanism of CTL inactivation by free peptide. The data indicate that the minimal requirements for free peptide to inactivate CTL are simultaneous occupancy of the class I MHC molecule and the TCR on the same CTL. This may occur in an isolated cell when the TCR of a cell binds the peptide–MHC complex formed on the same cell (A). Because the cell surface is not smooth, but has many projections and invaginations, TCR and MHC molecules on the same cell can easily come into contact. Alternatively, the same simultaneous engagement of TCR and MHC may occur between two cells. In the experiment shown in Fig. 5, in which two non–cross-reactive CTL lines were pulsed with each other's peptide, it can occur only when two cells of opposite types come together and recognize their peptides on the other cell's MHC molecules (B). However, in the simpler situation with a single CTL and a single free peptide, two sister cells each binding the peptide may inactivate each other (C). Whether the upper single-cell or the lower two-cell mechanism predominates may depend on cell density.

Simultaneous signaling though the TCR and MHC molecule on the same T cell appears to inhibit.

Recently, signaling though MHC class I, such as by cross-linking with specific antibodies, has been shown to regulate signal transduction though the TCR–CD3 complex and can lead to inhibition of cellular function (43), including cytotoxicity (44), perhaps by prolonging the duration of a CD3-induced elevation in intracellular Ca^{2+} (45). Sustained increases in intracellular Ca^{2+} can often lead to unresponsiveness in CTL (46); consequently, MHC class I modification of TCR–CD3 signals may represent another mechanism for the induction of anergy. T cell signaling through the class I molecule does not depend on the MHC cytoplasmic domain (47), but instead requires association of class I protein with other cell surface molecules (48, 49). Most studies of MHC class I signal transduction and regulation of CTL effector function have involved the use of antibodies as an MHC cross-linking agent. In our system, engagement of MHC class I–peptide complex on a CTL instead by a TCR may lead to anergy via MHC class I regulation of TCR signaling events. This mechanism of anergy induction appears to be distinct from that of TCR triggering in the absence of a costimulatory signal (19, 50).

The requirement for simultaneous signaling through the TCR and MHC molecule on the same CTL is reminiscent of the molecular mechanism proposed for the veto phenomenon (23, 24). The TCR of the CTL being vetoed must bind the MHC molecules of the veto cell, and the CD8 molecule of the veto cell binds the α3 domain of class

I MHC molecules of the CTL. Thus, the CTL has both its TCR and its class I MHC molecules engaged. The dual engagement permitted by free peptide may be a stronger signal through the MHC molecule, involving the interaction of TCR rather than CD8 with the peptide–MHC complex, and thus may account for inhibition of T cell clones resistant to classic veto. Nevertheless, the requirement for concurrent engagement of both molecules is a clear parallel. We are not aware of any previous connection made between free peptide inhibition and the veto phenomenon, but on the basis of the results presented here, we propose that free peptide inhibition of CTL activity is actually a process of self-veto.

There are a number of potential mechanisms of inhibition of virus-specific CTL in HIV-1–infected patients. We have observed a similar inhibitory effect of free peptide in vivo in primed animals (Takahashi, H., and Y. Nakagawa, unpublished observations), and Walden and Eisen (8) also observed a similar loss of CTL activity in spleen cells of primed animals after injection of an OVA peptide. Perhaps when virally infected cells are lysed and the digested intracellular proteins released into the environment of the T cell, anergy may be induced and clearance of the virus from other infected cells prevented. Thus, the self-veto mechanism shown here may contribute to inactivation of virus-specific CTL in vivo and virus persistence, and, conversely, restoration of such inactivated CTL may prevent virus spread and disease progression.

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