Role of Antigen, CD8, and Cytotoxic T Lymphocyte (CTL) Avidity in High Dose Antigen Induction of Apoptosis of Effector CTL

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Summary

Experimental data suggest that negative selection of thymocytes can occur as a result of supraoptimal antigenic stimulation. It is unknown, however, whether such mechanisms are at work in mature $CD8^+$ T lymphocytes. Here, we show that $CD8^+$ effector cytotoxic T lymphocytes (CTL) are susceptible to proliferative inhibition by high dose peptide antigen, leading to apoptotic death mediated by TNF-α release. Such inhibition is not reflected in the cytolytic potential of the CTL, since concentrations of antigen that are inhibitory for proliferation promote efficient lysis of target cells. Thus, although CTL have committed to the apoptotic pathway, the kinetics of this process are such that CTL function can occur before death of the CTL. The concentration of antigen required for inhibition is a function of the CTL avidity, in that concentrations of antigen capable of completely inhibiting high avidity CTL maximally stimulate low avidity CTL. Importantly, the inhibition can be detected in both activated and resting CTL. Blocking studies demonstrate that the CD8 molecule contributes significantly to the inhibitory signal as the addition of anti-CD8 antibody restores the proliferative response. Thus, our data support the model that mature $CD8^+$ CTL can accommodate an activation signal of restricted intensity, which, if surpassed, results in deletion of that cell.

The induction and maintenance of tolerance has been shown to significantly limit the potentially deleterious responses of self-reactive T lymphocytes. Although thymic education is thought to contribute significantly to the eradication of self-reactive T cells (1, 2), the induction of tolerance via peripheral exposure to antigen has been well documented (3–9). Deletion and/or inactivation of peripheral, antigen-reactive T lymphocytes has been observed in a number of experimental systems, including oral administration of antigen (10), repeated immunization with antigen (9), exposure to superantigens (8), and tissue-specific expression of antigens (5, 11). In addition, a recent report of clonal exhaustion has demonstrated the selective deletion of virus-specific T cells after challenge with high doses of virus (12). This result adds to a growing body of literature that examines the induction of tolerance in the presence of high doses of antigen. Several mechanisms have been postulated to explain this phenomenon including downregulation of cell-surface receptors leading to anergy (7, 11) and deletion of reactive cells (7, 8, 12).

The induction of peripheral tolerance via administration of high doses of antigen is poorly understood. In vitro data

investigating the role of high concentrations of antigen on the regulation of resting $CD4^+$ T lymphocytes have demonstrated a suppression of the proliferative response and induction of anergy in $CD4^+$ T cell clones when exposed to supraoptimal antigen doses (13). More recent data from Lenardo and collaborators using TCR transgenic animals specific for a myelin basic protein (MBP)[1] peptide, Ac1-11, demonstrated $CD4^+$ T cell deletion as a result of supraoptimal peptide/MHC stimulation (14). Transfer of $CD4^+$ cells from the transgenic mice into nontransgenic mice induced experimental allergic encephalomyelitis in the recipient animal. However, recipient mice treated with high doses of MBP peptide did not develop experimental allergic encephalomyelitis. In vitro studies showed that such high antigen conditions resulted in the deletion of recently activated cycling MBP-reactive T cells via activation-induced cell death (AICD), thereby establishing tolerance. AICD is thought to result from reexposure of cycling T

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[1] Abbreviations used in this paper: AICD, activation-induced cell death; MBP, myelin basic protein.

lymphocytes to antigen (14). Although characterized primarily in CD4$^+$ T cells, AICD has also been observed in bulk lymph node CD8$^+$ T cells stimulated with anti-CD3 (15), but not in CTL lines of defined epitope specificity.

Previously, we have demonstrated that CD8$^+$ CTL lines of differing avidity can be selected by varying the peptide/MHC determinant density on APC (16). In this system, selection of low avidity CTL lines by high determinant density should be possible only if high avidity CTL are being actively deleted. In the current report, we have investigated the effect of high dose antigen on CTL of differing avidities to define parameters giving rise to negative selection in mature, peripheral CD8$^+$ effector T lymphocytes. Classically, the functional consequence of TCR engagement by CD8$^+$ CTL has been evaluated by the lysis of appropriate target cells, and under these circumstances, high dose inhibition has not been observed (17). As little is known about the effects of stimulation with supraoptimal antigen on the proliferative response of either resting or cycling CD8$^+$ CTL, CTL lines of either high or low avidity (as measured by their dose response curve to peptide antigen) were examined for their proliferative response to APC pulsed with a range of peptide concentrations. The data presented herein demonstrate that CD8$^+$ CTL can undergo inhibition and cell death in a peptide specific and dose titratable manner, mediated at least in part by the release of TNF-α, but not by Fas. In addition, the susceptibility of CTL to inhibition at any given peptide concentration is dependent on the avidity of those CTL. Importantly, the observed inhibition appears to be independent of the initial activation state of the CTL, and is set in motion even in resting CTL within the first 9 h of stimulation, in contrast to AICD in CD4$^+$ T cells. Thus, CD8$^+$ CTL, depending on their avidity, are susceptible to negative regulation in the periphery by supraoptimal peptide/MHC determinant density on APC.

Materials and Methods

Mice and Antibodies. BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Anti-CD8α antibody (clone 53-6.7), Jo2 anti-fas antibody, and FcBlock were purchased from Pharmingen (San Diego, CA). Neutralizing anti–TNF-α polyclonal antiserum was purchased from Genzyme (Cambridge, MA). Activity of the anti-Fas antibody to inhibit Fas–Fas ligand interaction was measured as described (18; see legend to Fig. 4).

Peptides. Peptides were synthesized on an automated peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using t-boc chemistry (19). The peptides were cleaved from the resin with HF and initially purified by size exclusion chromatography (P4 Biogel; Bio-Rad Laboratories, Mountain View, CA). Purification to single peaks was achieved by reverse-phase HPLC on μbondapack reverse-phase C18 analytical and preparative columns (Waters Associates, Milford, PA). I10 (RGPGRAFVTI) is the immunodominant epitope from gp160 in BALB/c mice (20–22).

Generation of CTL Lines. 7.5 $\times$ 10^6 responding BALB/c spleen cells from mice previously immunized with a recombinant vaccinia expressing the gp160$_{\text{IIIB}}$ protein from HIV-1 were cocultured with 3.5 $\times$ 10^6 stimulating BALB/c splenocytes (3,000 rad)

pulsed with various concentrations (100, 0.1, or 0.0001 μM) of I10 peptide or in the presence of 1 μM free peptide in a 24-well plate containing 2 ml of a 1:1 mixture of RPMI 1640 and Eagle Hank's amino acid (EHAA) medium supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 5 $\times$ 10^{-5} M β-mercaptoethanol, 10% FCS, and 10% T-stim (Collaborative Biomedical Products, Bedford, MA). CTL lines were established from primary cultures and were maintained by weekly restimulation of 3–5 $\times$ 10^5 cells/well in the presence of 5 $\times$ 10^6 irradiated (3,000 rad) BALB/c spleen cells pulsed with the appropriate concentration of I10 peptide.

^{51}Cr-release Assay. The ^{51}Cr release assay was carried out as previously described (23). 10^6 target cells were labeled with 300 μCi of Na$_2$ ^{51}CrO$_4$ in 200–250 μl for 2 h at 37°C. In some cases, targets were pulsed with 10 μM peptide during labeling. Cells were then washed three times and added at 3,000 cells/well along with the appropriate number of effector cells in 96-well round-bottom plates. In some cases, peptide was added directly in the CTL assay in the absence of previous pulsing. After 4 h, supernatants were harvested and counted in an Isomedic gamma counter (ICN, Horsham, PA). The mean of the triplicate samples was calculated, and the percent of ^{51}Cr release was calculated according to the following equation:

$$percent\ specific\ ^{51}Cr\ release = 100 \times [(experimental\ ^{51}Cr\ release - control\ ^{51}Cr\ release)/(maximum\ ^{51}Cr\ release - control\ ^{51}Cr\ release)],$$

where experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents target cells mixed with medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from target cells exposed to 2.5% Triton X-100.

Proliferation Assays. CTL (day 4 [d4] or d11 after stimulation) were plated at 5 $\times$ 10^4/well in a 96-well round bottom microtiter plate. Where appropriate, anti-CD8 antibody was added and plates incubated at 37°C for 15 min. After this incubation, irradiated (3,000 rad) BALB/c splenocytes previously pulsed with I10 peptide and washed three times were added at 3 $\times$ 10^5/well. Supernatant from the final wash of stimulators pulsed with 100 μM I10 peptide was reserved and added to wells with 0.001 μM-pulsed stimulators at a final dilution of 1:1 to ensure that effects seen with 100 μM-pulsed stimulators was not caused by residual, free I10 peptide that could bind the CTL directly. In some cases, Jo2 anti-Fas (10 μg/ml) in combination with FcBlock (5 μg/ml), both from Pharmingen, or anti–TNF-α (1:50) antibodies (Genzyme) were used. FcBlock was added to prevent possible crosslinking of anti-Fas by APC. Proliferation was measured by addition of 1 μCi [^{3}H]thymidine/well at 0, 24, or 48 h, and plates were harvested at 24, 48, or 72 h, respectively. Results obtained at all harvest times were qualitatively similar. Results are the geometric mean of triplicate cultures.

Apoptotic Death Assays. CTL were enriched by centrifuging lines over Ficoll. After washing, 10^6 CTL were added along with 5 $\times$ 10^6 peptide-pulsed stimulators (depleted of Thy 1.2$^+$ cells) in 2 ml of medium, containing 10% T-stim as a source of IL-2, per well of a 24-well plate. After 46 h, cells were harvested and incubated with biotin-conjugated Thy 1.2 mAb, followed by PE-avidin or FITC-conjugated Thy 1.2 to identify CTL, washed, resuspended in 50% FCS in PBS, and permeabilized by addition of three parts 70% EtOH. After overnight fixation at 4°C, cells incubated with Thy 1.2–biotin and PE-avidin were washed with cold PBS and incubated for 30 min with 5 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) at 37°C. Cells were then centrifuged, and the pellet was resuspended in 20 μl PBS. Apop-

totic nuclei were identified by fluorescent microscopy (24). Alternatively, EtOH fixed cells were washed and incubated with RNAse A for 30 min at 37°C, followed by addition of propidium iodide (25). Cells were analyzed on a FACSort® (Becton Dickinson & Co., Mountain View, CA). The percentage of Thy 1.2+ cells with hypodiploid DNA was used as a measure of apoptotic death as in (25).

Results

CTL Lines with Distinct Avidities Are Differentially Inhibited by Stimulators Presenting High Dose Antigen. CTL lines were generated by stimulation with splenocytes pulsed with either high dose (100 μM) or low dose antigen (0.0001 μM) (16). The two lines are distinct in their determinant density requirements for both proliferation (Fig. 1 *A*) and CTL lysis, and are stable in their respective phenotype (16). The concentration of peptide antigen required for optimal proliferation varied by nearly 1,000-fold between the high and low avidity lines (Fig. 1 *A*). CTL were tested on d4 after routine stimulation and assays were done in the presence of 10% T-stim as a source of IL-2, as required for proliferation by CD8+ cells in response to antigen. Fig. 1 *A* shows that in the presence of APC prepulsed with 100 μM I10 peptide, the higher avidity CTL (generated on 0.0001 μM–pulsed stimulators) were completely inhibited in their proliferative response. The inhibition was CD8 dependent, since proliferation could be at least partially restored by the inclusion of anti-CD8 antibody (Fig. 1 *B*). Further, the restoration was dose dependent in that decreasing amounts of anti-CD8 antibody had a diminishing ability to block the inhibition. The elegant interplay of CD8 with TCR receptor signaling is evident in the contribution by anti-CD8 at doses of antigen that are optimal for activation, as measured by proliferation (e.g., 0.001 μM), where the presence of anti-CD8 antibody now blocks proliferation by up to 40%. Thus, the contribution of CD8 enhances either inhibition or activation of the CTL, depending on the amount of antigen present on the stimulator. In contrast to the high avidity line, the low avidity line is not susceptible to inhibition by the same high antigen dose (Fig. 1 *A*). In fact, concentrations of peptide antigen that are inhibitory for the high avidity line promote optimal proliferation of the low avidity line. Furthermore, proliferation of this line at all peptide doses, even 100 μM, is susceptible to blocking by anti-CD8 antibody (Fig. 1 *B*).

To interpret the data, it is important to determine that the inhibition observed for the high avidity line is not caused by residual free peptide in the cells pulsed with high dose antigen, which can then bind to and be presented by MHC molecules on the CTL. Such inhibition by self-presentation of peptide has been shown, in some cases, to result in CTL fratricide, suicide, or anergy (26–30). The inability of supernatant from the final wash of the 100 or 10 μM–pulsed cells to cause inhibition when added to wells containing 0.001 μM I10–pulsed stimulators shows that the inhibition is caused solely by presentation of the relevant
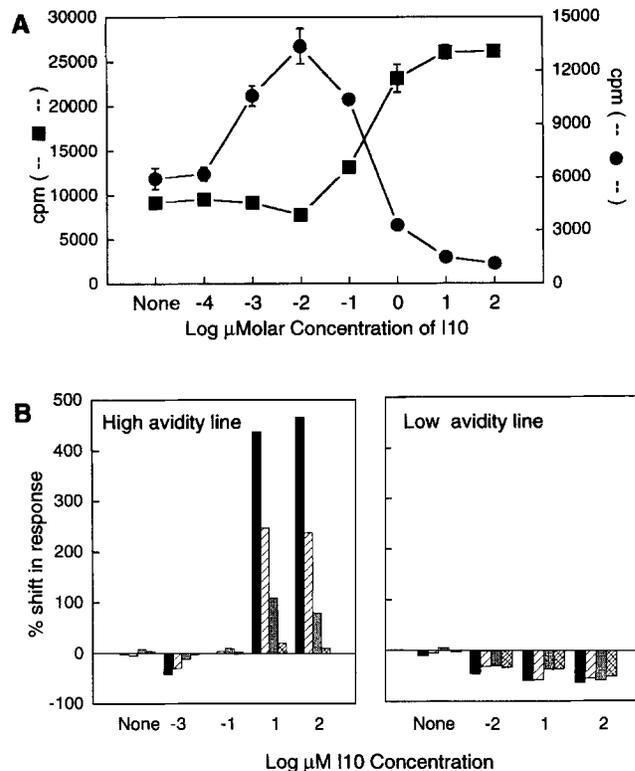

Figure 1. The d4 high avidity line is inhibited in its proliferative response to APC pulsed with high concentrations of peptide, and this inhibition can be reversed by anti-CD8 antibody. (*A*) The high and low avidity CTL lines were assayed for proliferation on day 4 after routine stimulation. CTL were stimulated with APC pulsed with various concentrations of peptide. Cultures were pulsed with [³H]thymidine at 48 h and harvested at 72 h. APC pulsed with 100 or 10 μM peptide inhibited proliferation of the high avidity line (*circle*) while maximally stimulating the low avidity line (*squares*). As a control for residual free peptide, supernatant from the final wash of the 100 and 10 μM–pulsed APC was added at 1:1 dilution into wells with 0.001 μM–pulsed APC. Counts from addition of the 100 μM–pulsed APC wash were 9,174 ± 270 and from the 10 μM–pulsed wash APC were 9,982 ± 409, i.e., no inhibition relative to wells in the absence of the wash supernatant. (*B*) The addition of soluble anti-CD8 antibody partially restored the proliferative response of the high avidity line while blocking the response of the low avidity line. Antibody was added at four concentrations: 20 μg/ml (*solid bars*), 5 μg/ml (*striped bars*), 1.3 μg/ml (*stippled bars*), and 0.32 μg/ml (*cross-hatched bars*). The percentage of shift in response was calculated as 100× (cpm in the presence of anti-CD8 − cpm in the absence of anti-CD8)/cpm in the absence of anti-CD8.

peptide ligand on APC and not to residual free peptide (see figure legend).

To ensure that the lack of response observed with high dose antigen was not the result of very early proliferation that was complete by the time of pulsing with [³H]thymidine, a kinetic experiment was performed. Inhibition by high dose antigen was observed even during the 0–24-h period (data not shown). This suggests that the inhibition observed was not the result of a shift in the kinetics of DNA synthesis relative to the time of the pulse, but instead, is a relatively immediate arrest of the proliferative response of the high avidity CTL.

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Inhibition by High Dose Antigen Does Not Depend on Recent Activation of the CTL. Previous studies have demonstrated the importance of cell cycling for cell death resulting from exposure to high dose antigen (14) or high concentrations of anti-TCR antibody (31, 32). Thus, the high and low avidity lines were analyzed for susceptibility to high dose inhibition at a time point when they should not be actively cycling (d11 after routine stimulation). As observed with activated d4 CTL, the high avidity resting CTL were unresponsive to stimulators pulsed with high dose antigen (100 and 10 μM) (Fig. 2 *A*). Furthermore, the presence of anti-CD8 antibody could relieve the inhibition and allow for antigen-specific proliferation (Fig. 2 *B*). Again, in agreement with results obtained with d4 CTL, the low avidity CTL line was maximally stimulated by APC pulsed with high dose antigen (100 and 10 μM), and the proliferation was dependent on CD8 interaction at all peptide concentrations tested (Fig. 2, *A* and *B*). Thus, we find that CD8$^+$ CTL are susceptible to high dose inhibition independent of recent activation and cell cycling.

High Dose Peptide Antigen Induces Apoptotic Death of the CTL. The observed inhibition of the proliferative response to high dose antigen could be the result either of

Figure 2. Resting d11 high avidity CTL are inhibited in the their proliferative response to high dose antigen. (*A*) The high and low avidity CTL lines that were d11 after routine stimulation were tested as described in Fig. 1. Both activated (Fig. 1) and resting high avidity CTL were susceptible to high dose inhibition. (*B*) As with d4 high avidity CTL, anti-CD8 antibody allowed response to high dose antigen by d11 CTL. Percentage of shift in response was calculated as described in Fig. 1.

anergy or cell death. To determine the mechanism of the reduction in the proliferative response of high avidity CTL, lines were stimulated with 0.001 or 100 μM peptide-pulsed splenocytes depleted of Thy 1.2$^+$ cells. Depleted stimulators were required for discrimination of CTL during analysis. 45 h after stimulation, cultures were harvested, incubated with biotinylated anti-Thy 1.2 Ab followed by PE-avidin (to detect CTL), and fixed overnight. Samples were then incubated with Hoechst 33342 nuclear stain. When viewed by fluorescence microscopy, CTL could be distinguished from APC by red membrane staining. The number of CTL with apoptotic nuclei, known to exhibit specific morphological changes including condensation of DNA, could then be quantitated. Fig. 3 *A* shows that high avidity CTL stimulated with APC pulsed with 100 μM peptide are undergoing significant apoptosis compared with CTL stimulated with 0.001 μM–pulsed APC (47 vs. 3%). No apoptotic death was observed with the low avidity line under identical conditions (Fig. 3 *A*), although both high and low avidity lines were susceptible to apoptotic deletion using high concentrations of immobilized anti-CD3 antibody (data not shown). Results similar to those observed with d4 CTL were obtained with CTL that were 11 d after routine stimulation exposed to APC pulsed with high versus low concentrations of antigen (data not shown). Thus, regardless of the activation state of the CTL, the result of exposure to high dose antigen is identical and occurs with the same kinetics. In addition to this analysis, CTL undergoing apoptotic death were quantitated by hypodiploid DNA analysis. Results from these analyses were in agreement with results obtained by Hoechst analysis (Fig. 3 *B*). Apoptotic death of the CTL line was found to correlate with inhibition of proliferation.

When tested for cytolytic activity, high avidity CTL could readily lyse target cells pulsed with high concentrations of peptide (data not shown). As shown above, however, these concentrations of peptide cause inhibition and death. These two events can be reconciled by examining the kinetics of death. Apoptotic nuclei are not evident at 24 h, but require 40–48 h to appear (data not shown). Thus, there is adequate time for the earlier event of cytolysis, which occurs within 4 h after exposure to antigen-pulsed targets, to take place before the ultimate death of the CTL at a later time point.

Inhibition Can be Prevented by the Presence of Anti–TNF-α Antibody. Recent evidence suggests that TNF-α can mediate apoptotic death of CD8$^+$ T lymphocytes that are undergoing apoptotic death as a result of hyperengagement of the TCR of recently activated cells via anti-TCR antibodies (15). To determine whether TNF-α is involved in the inhibition observed in our cultures, an anti–TNF-α polyclonal antibody was added to CTL stimulated with either high or low dose antigen-pulsed stimulators. As shown in Fig. 4, the presence of anti–TNF-α antibody restored the proliferative response of high avidity CTL stimulated with supraoptimal peptide/MHC determinant density while having no effect on the response of the low avidity CTL line to the same stimulators. Fas cross-linking, shown in
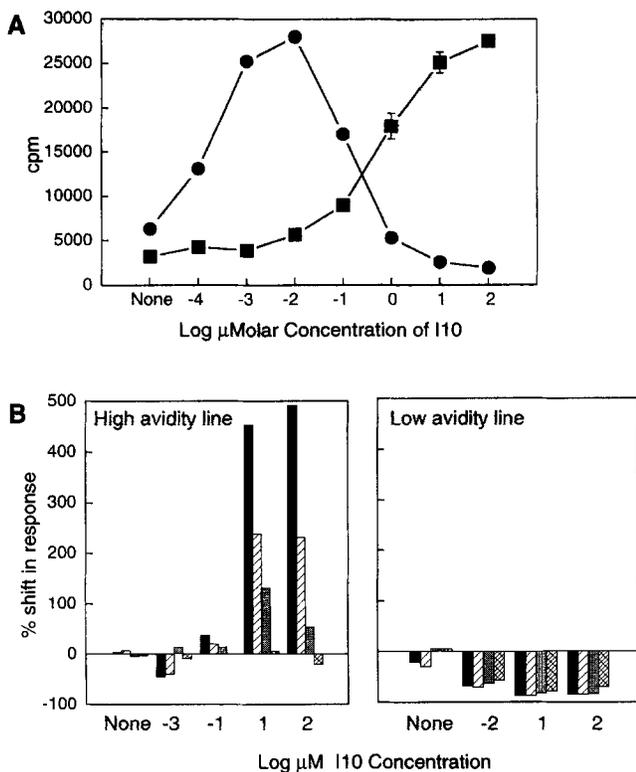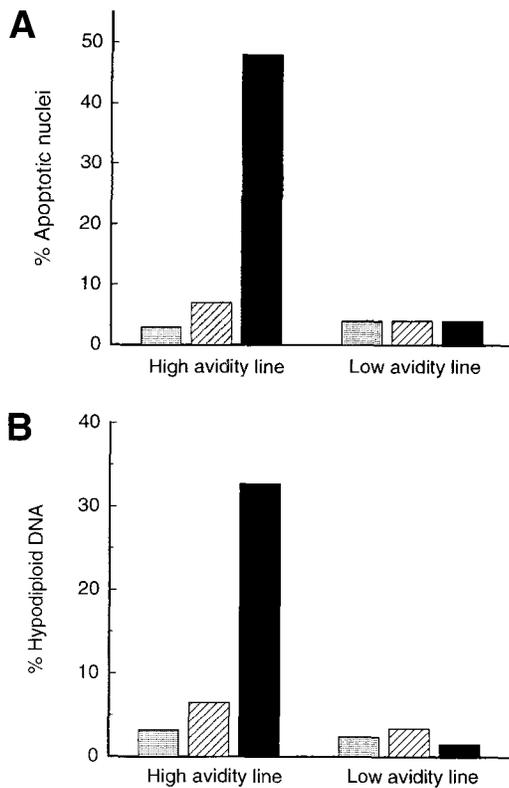
A
% Apoptotic nuclei (0–50)
High avidity line / Low avidity line

B
% Hypodiploid DNA (0–40)
High avidity line / Low avidity line

Figure 3. The lack of a proliferative response by the high avidity CTL to high dose antigen is the result of apoptotic death. 4 d after routine stimulation, high and low avidity CTL were analyzed for apoptotic nuclei (A) or hypodiploid DNA (B) 45 h after exposure to high dose or low dose antigen. APC (Thy 1.2-depleted splenocytes) were either unpulsed (*stippled bars*), or pulsed with 0.001 µM (*striped bars*), or 100 µM (*solid bars*) peptide. For analysis of apoptotic nuclei, CTL were identified by incubation with biotinylated-Thy 1.2 and PE-avidin. Cells were then fixed, and Hoechst dye was added to view the nucleus. CTL were assessed for apoptotic nuclear morphology by fluorescence microscopy. As a control for residual free peptide, supernatant from the final wash of the 100 µM–pulsed APC was added at 1:1 in some wells with 0.001 µM–pulsed APC and 14% of CTL contained apoptotic nuclei. For hypodiploid DNA analysis, CTL were identified by incubation with FITC-conjugated antibody specific for Thy 1.2. Propidium iodide was added for DNA detection.

A
cpm (0–8000)
100 / 0.001

B
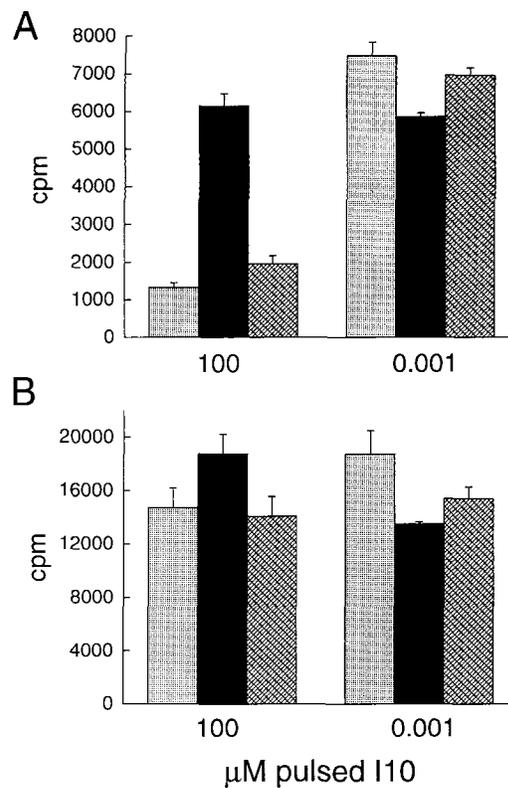cpm (0–20000)
100 / 0.001
µM pulsed I10

Figure 4. Neutralizing anti-TNF-α antibody can reverse the inhibition obtained with supraoptimal peptide/MHC determinant density. 4 d after routine stimulation, high (A) and low (B) avidity CTL were assayed for proliferation resulting from stimulation with irradiated splenocytes pulsed with various concentrations of peptide antigen, as described in Fig. 1 A. No antibody (*stippled bars*), neutralizing anti-TNF-α antibody, 1:50 (*solid bars*), or Jo2 anti-Fas antibody (10 µg/ml; *cross-hatched bars*) was added to determine the role of these two molecules in the inhibition observed. In the case of the anti-Fas antibody, FcBlock (5 µg/ml) was added to prevent possible cross-linking of the antibody by APC. (In other experiments, the presence of FcBlock alone did not affect the response.) No significant effect was observed in the presence of anti-Fas antibody. As a positive control for the function of the anti-Fas antibody, we tested its ability to inhibit lysis of Fas-expressing L1210 transfectants by the Fas ligand–expressing d11S CTL hybridoma (18). At a 15:1 E/T ratio, the anti-Fas antibody (10 µg/ml) inhibited specific ^{51}Cr release from 78 ± 2% in the absence of antibody to 15 ± 2% in the presence of the antibody (81% inhibition).

multiple studies to induce death of T lymphocytes, does not appear to be involved in the inhibition and death under the conditions tested here, since soluble anti-Fas antibody did not restore the proliferative response of the high avidity CTL (Fig. 4).

Discussion

This study reports the ability of high dose peptide antigen presented by APC to induce apoptotic death in a high avidity CD8^{+} CTL line while maximally activating CTL of lower avidity. This is the first report to document a role for increasing peptide ligand presented by APC in the deletion of mature CD8^{+} CTL, as well as the contribution of avidity in determining cell fate. These findings are distinct from previous studies of CD8^{+} CTL in which the effect of supraoptimal TCR stimulation via antibody or purified al-

loantigen was not assessed either with regard to the independent role of increasing peptide ligand density or with regard to the effects of such treatment on resting CTL (31, 33). The data presented in this study suggest that the number of peptide determinants presented by the APC regulates either positively or negatively the ability of CTL to proliferate in response to antigen. High avidity CTL, capable of being activated by a low number of peptide/MHC complexes, are deleted by exposure to APC with a very high density of peptide/MHC complexes, while low avidity CTL are maximally stimulated at this same high determinant density.

The ability of soluble anti-CD8 Ab to restore the proliferative response of high avidity CTL stimulated with 100

μM–pulsed APC suggests that the signal received by CTL is a summation of the interaction of a number of proteins, in this case, the TCR and CD8. If the combined signals from these two receptors reach a certain threshold, then cells are activated. If, however, the signal surpasses a higher threshold, above the range for activation, as is the case for high avidity CTL stimulated with APC pulsed with 100 μM peptide, death will result, due at least in part to the release of TNF-α. However, if the contribution of CD8 is blocked, then the lower level of signal generated results in CTL proliferation. In situations in which the number of peptide/MHC complexes is more limited, the level of signal delivered from TCR binding alone is insufficient to reach the threshold required for activation, and signal amplification via CD8 becomes a mandatory part of the interaction to stimulate proliferation. Thus, the engagement of CD8 and TCR is critical both for positive and negative regulation of CTL.

At first, the observed inhibition of the d4 CTL seems to be similar to the activation-induced death reported by others (14, 15, 31, 32, 34–38). It is thought that death via this pathway depends on the reexposure of recently activated cycling cells to one of a variety of stimuli (8, 14, 31, 32, 34–38). The inhibition of CD4$^+$ cell proliferation at high antigen concentration may be the result of a second encounter of the cells with antigen within 24 h of the initial stimulation, resulting in AICD (1, 14).

The data presented here suggest that both resting and activated CD8$^+$ CTL are equally susceptible to inhibition and apoptotic death by high concentrations of peptide antigen presented by MHC on the surface of APC. Similar to AICD, we observe apoptotic deletion in response to supraoptimal peptide/MHC determinant density. Before the present report, this had not been reported for peptide-specific, CD8$^+$ CTL. In addition, we have demonstrated that the mediator of death is, at least in part, TNF-α, in agreement with a recent study by Zheng et al. in which heterogeneous populations of CD8$^+$ lymph node T lymphocytes were stimulated with high doses of anti-CD3 antibody to induce AICD (15). In our study, the apoptotic death of the high avidity CTL line suggests that TNF-α is produced only after stimulation with very high peptide/MHC determinant density or that high determinant density is required to achieve levels of TNF-α production that are capable of inducing apoptotic death. Alternatively, the action of TNF-α may be attributable to differential TNF-α receptor regulation after stimulation with various antigen doses. Identification of TNF-α as the mediator of the apoptotic signal is likely to be the explanation for the requirement of 40–48 h before apoptotic death is measurable after stimulation with high concentrations of pulsed peptide antigen. Experiments are currently underway to determine whether this process is regulated by increased TNF-α production or TNF-α receptor upregulation or both.

Although the above similarities exist, several important differences from AICD are apparent. First, Fas and Fas ligand, which have been suggested to mediate classical AICD (39), do not play a role here (Fig. 4). Second, our

data demonstrate that high avidity, activated d4 CTL , and resting d11 CTL require 40–48 h for the appearance of apoptotic nuclei. As discussed above, for AICD to occur, resting CD4$^+$ cells are thought to require two distinct activation encounters with antigen, and it is the second engagement by the now cycling cell that is responsible for triggering death. Experiments with d11 CTL used in this study, in which 100 μM–pulsed APC were removed after 9 h of exposure and the CTL were replated in the presence of unpulsed APC, demonstrated that the commitment to death has already occurred during this initial antigenic encounter, suggesting that these CTL do not require a second encounter with antigen while cycling to induce apoptosis (data not shown). The only precedent for inhibition and apoptosis resulting from a single encounter with antigen is in the nonphysiological case of T cell hybridomas (40). Moreover, the same concentration of peptide is required for the deletion of both recently activated and resting CTL (Figs. 1 *A* and 2 *A*). Thus, there is no evidence that actively cycling CD8$^+$ CTL cells are more predisposed or sensitive to supraoptimal TCR engagement for induction of apoptotic death. Finally, as noted previously, proliferation of the CTL in response to high dose antigen is inhibited from the very beginning of the culture, as determined by a pulse with [^{3}H]thymidine between 0–24 h after initiation of the culture. Together, these data support the hypothesis that the signal resulting in apoptosis of resting CTL is a result of the first antigenic stimulation with supraoptimal determinant density. These data argue that the strength of signal is much more important in determining the outcome of TCR engagement by CD8$^+$ CTL effector lines than is the activation state of the cells prior to encounter with peptide/MHC.

Several studies have shown that CTL–CTL presentation of peptide can cause inhibition of lytic function, anergy, or death (26–30). However results obtained using T cell–depleted APC or addition of control supernatant from the final wash of 100 μM–pulsed APC rule out the possibility of T cell–T cell antigen presentation or free peptide inhibition in our system.

The data presented herein may help explain results by Moskophidis et al., which suggest that CTL capable of viral clearance undergo deletion as a result of clonal exhaustion after challenge with large doses of virus (12). Under such circumstances, viral antigen may be either constitutively presented for extended periods of time or presented at an increased level at the cell surface on a per cell basis. Our results suggest that the latter would result in the deletion of high avidity CTL, which are more effective for viral clearance, leaving only low avidity CTL inadequate to control viral spread (16, 41). Moreover, it has been demonstrated that during the course of HIV-1 infection, there is an ongoing loss of HIV-specific CD8$^+$ T cells (42). Given the viral load attained during HIV-1 infection, it is possible that a determinant density is achieved which is capable of inducing deletion of the virus-specific cells, in a manner analogous to the deletion reported in this study.

Our previous finding (16) that only low avidity CTL are

expanded under conditions of high dose antigen are explained by the data presented here. Under such conditions, high avidity cells would undergo apoptosis as a result of supraoptimal engagement of the TCR, and thus be deleted from the line.

In conclusion, the delivery of an activating signal to CTL is complex and several proteins are likely to contribute. Preeminent among these are the TCR and CD8. The number of peptide/MHC complexes required for optimal TCR engagement is a function of the inherent requirements of the CTL in that there is a specific range of determinant densities that is acceptable for activation and concentrations of ligand that exceed this range will induce deletion of the CTL, mediated at least in part by the production of TNF-α, and not by Fas–FasL interaction. In addition, susceptibility to high dose inhibition by the CTL is not restricted to recently activated cells. The deletion reported here may contribute to high zone tolerance and/or clonal exhaustion, and loss of HIV-specific CTL during progression toward AIDS. Moreover, this knowledge may have implications for therapy of autoimmune diseases or allograft rejection.

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