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T:T Antigen Presentation by Activated Murine CD8+ T Cells Induces Anergy and Apoptosis

Jian-Guo Chai,* Istvan Bartok,† Diane Scott,‡ Julian Dyson,† and Robert Lechler2*

Using an IL-2-secreting, noncytolytic, H-Y-specific, CD8+ T cell clone, the functional consequences of Ag presentation by T cells to T cells were investigated. Incubation of the T cells with H-Y-soluble peptide led to nonresponsiveness to Ag rechallenge. This was due to the simultaneous induction of apoptosis, involving approximately 40% of the T cells, and of anergy in the surviving cells. These effects were strictly dependent upon bidirectional T:T presentation, in that exposure of C6 cells to peptide-pulsed T cells from the same clone induced proliferation but not apoptosis or anergy. The inhibitory effects of T:T presentation were not due to a lack of costimulation, since the T cells expressed levels of CD80 and CD86 higher than those detected on cultured dendritic cells. These effects were not reproduced by CD80 or CD86 Ab. Neither Fas nor TNF-α was expressed/produced by the C6 cells, and coligation of MHC class I molecules and TCR failed to reproduce the effects of T:T presentation. Taken together, these data suggest that T:T Ag presentation induces anergy and apoptosis in murine CD8+ T cells and may reflect the regulatory consequences of T:T interactions in the course of clonal expansion in vivo.


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Materials and Methods

Animals

CBA/Ca and BALB/c mice were purchased from Harlen, Olac (Bicester, U.K.) and were used at 8 to 12 wk of age. H-Y-specific TCR transgenic mice were described in detail previously (28).

T cell clones and peptide

C6 (29), an H-Y-specific, CD8+ T cell clone, recognizes an H-Y Ag peptide TENSNGKDI (30) (called H-Y Kk peptide) presented by the MHC class I molecule Kk. C6 cells (1–2 × 10^5/well) were routinely maintained in...
culture by stimulation with irradiated CBA male splenocytes (5 × 10^7 well) and rhIL-2^1 (10 U/ml) in 24-well plates (Costar, Cambridge, MA) every 3 wk. Ten to fourteen days after their last stimulation, viable C6 cells were isolated by density centrifugation on Lympho-Sep (Sera-Lab, Sussex, U.K.). To remove any remaining splenic accessory cells before the use of C6 in functional assays, the cells were subjected to low speed centrifugation (50 × g) and twice washed. Immunoreactive MHC class II-expressing cells were present. IF8 is an allospecific CD4^+ T cell clone (31).

Abs, fluorescence dye, and mitogens

The following mAbs (in the form of either hybridoma culture supernatants or purified Abs) were used in the present study: anti-H-2^A (10.2.16; TIB-93, ATCC, Rockville, MD), anti-H-2^D (14.4.4S; HB-32, ATCC), anti-H-2^E (MK-D6; HB-3, ATCC), anti-CD3 (145-2C11; CRL-1975, ATCC), anti-IL-4 (11B11; HB-188, ATCC), anti-CD4 (YTS 191) (32), anti-CD8 (YTS 169) (32), anti-Thy1.2 (30-H12; TIB107, ATCC), anti-B220 (RA3-3B1/61; TIB-146, ATCC), anti-D^3 (28-14-85; HB-27, ATCC), anti-K^b (16-3-1N; HB-25, ATCC), and anti-K^d (12-2-25; HB-50, ATCC). Murine CTLA-4 (3H10; protein 31) was purchased from protein G-Sepharose. Purified anti-CD80 (1G10), anti-CD86 (GL1), anti-Fas (Jo2), and anti-FasL antibody preparations (San Diego, CA). Anti-CD28 (39.51) (34), anti-CTLA-4 (UC11-4F10 –11) (11B10; HB-188, ATCC), and anti-K^k D^k (12-2-2S; HB-50, ATCC). Murine IFN-γ (10.2.16, 14.4.4S, and YTS169 to purify CBA CD8^+ T cells; GM-CSF-1, granulocyte/macrophage colony-stimulating factor-1.

SUPPLEMENTED WITH 5% FCS, 50 μM 2-ME, 100 U/ml penicillin, 10 μg/ml streptomycin, and 2 mM L-glutamine containing 5% culture supernatant (v/v) from a GM-CSF-secreting transfected cell line (provided by Dr. D. Gray). On day 3 of culture, nonadherent cells were removed by gentle pipetting. Fresh GM-CSF-containing culture medium was added to the dishes. The maximum yield of DC was obtained between days 6 and 8 of culture. The nonadherent fraction. The yield and purity of DC were assessed by flow cytometric analysis (double staining with CD11c and MHC class II). DCs were used as syngeneic accessory cells or allogeneic stimulators in Con A responses or allogeneic MLR, respectively.

T cell proliferation assays

RPMI 1640 medium supplemented with 10% FCS (GibcoBachem, Esher, U.K.), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-ME was used as complete culture medium in all T cell proliferation assays. T cells were routinely cultured in 96-microwell plates in a volume of 0.2 ml for 3 days. Two days later, 1 μCi of [*H]*thyminidine (ICN, Costa Mesa, CA) was added to each well, and T cells were harvested onto glass-fiber filters using a Wallac 1295-004 Betaplate 96-well harvester (Wallac Oy, Turku, Finland) after an additional 24 h. [*H]*Thymidine uptake was measured using a Wallac 1205 Betaplate liquid scintillation counter (Wallac Oy). These results are expressed as mean or Δ (corrected for background counts) counts per minute for triplicate cultures. SEs were routinely <10%.

Con A responses and allogeneic MLR

Purified CD4^+ T cells (5 × 10^5/well) from CBA female mice were stimulated with Con A (1–8 μg/ml) in the presence of syngeneic accessory cells (DC, 5 × 10^5/well; C6 and adherent cells, 5 × 10^5/well) in a 96-well plate. Allogeneic MLRs, CD8^+ T cells (1 × 10^5/well) from BALB/c female mice were incubated with the indicated numbers of allogeneic CBA stimulator cells (irradiated DC, C6, or adherent cells; 5 × 10^5/well to 10^6/well) in round-bottom 96-well plates for 3 days. Cultures were pulsed with [*H]*thymidine and harvested as described above.

Peptide-induced proliferation in resting transgenic CD8^+ T cells

For the measurement of primary Ag-specific responses, CD8^+ T cells were purified from H-Y-specific TCR-transgenic female mice and were used as responders (5 × 10^5/well). C6 cells, splenic adherent cells, or purified CD4^+ T cells from CBA female mice were prepulsed with peptide and used as stimulators (5 × 10^5/well). For peptide pulsing, cells were incubated with peptide for 2 h (C6 cells) or overnight (adherent cells or CD4^+ T cells); the cells were then washed, irradiated, counted, and used as stimulators. Cultures were pulsed with [*H]*thymidine and harvested as described above.

Peptide-induced proliferation in C6 cells

C6 cells (1–2 × 10^5/well) were cultured with soluble peptide in the absence or the presence of irradiated CBA female splenocytes (5 × 10^5/well) or in the absence or the presence of the indicated mAbs or their Fab in flat-bottom 96-well plates for 3 days. In some experiments, C6 cells (1 × 10^6/well) were stimulated with peptide (5 × 10^5/well), peptide-prepulsed C6 (5 × 10^5/well), or peptide-prepulsed C6 (5 × 10^5/well) in flat-bottom 96-well plates for 3 days. In rechallenge cultures, peptide-treated or untreated C6 cells (1 × 10^5/well) were stimulated with peptide-prepulsed CBA female splenocytes (5 × 10^5/well) in the absence or the presence of anti-CTLA-4 (10 μg/ml) in flat-bottom 96-well plates for 3 days.

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^1 Abbreviations used in this paper: rhIL-2, recombinant human IL-2; ATCC, American Type Culture Collection; Fasl., Fas ligand; PI, propidium iodide; DC, dendritic cell; GM-CSF-1, granulocyte/macrophage colony-stimulating factor-1.
Measurement of cytokines

Culture supernatants harvested from the rechallenge cultures were tested for IL-2 content using CTLL-2 cells as indicator cells (TIB214, ATCC) in the presence of neutralizing anti-IL4 mAb 11B11 (HB-188, ATCC). The growth of CTLL-2 cells was measured by [3 H]thymidine uptake as described for T cell proliferation assays. TNF-α activity in culture supernatants harvested from the primary cultures was measured using an ELISA kit (Endogen, Cambridge, MA).

Flow cytometric analysis of phenotype and apoptosis in C6 cells

The purity of CD4+ or CD8+ T cells was examined by staining with FITC-conjugated anti-CD3, anti-CD4, and anti-CD8 mAbs. The phenotype of C6 cells was tested by direct staining for CD3, CD8, CD25 (IL-2R), and Vβ11 or by indirect staining for CD28, CTLA-4, CD80, CD86, Kα, Fas, Fasl, and membrane-bound TNF-α. After the rest period, untreated and peptide-treated C6 cells were stained again by anti-Vβ11-FITC or control mAb to examine TCR modulation. As described previously (17), apoptosis was measured by PI staining and flow cytometric analysis. Briefly, C6 cells (1 x 10^5/well) were cultured with medium or 100 nM peptide overnight, and the cells were washed and rested for another day in fresh medium containing 10 µg/ml of anti-Kα mAb (HB25). Then these peptide-untreated and peptide-treated C6 cells (1 x 10^5/well) were restimulated with peptide-prepulsed CBA female splenocytes (5 x 10^7/well) in the presence of exogenous rhIL-2 (10 U/ml) for 3 days. Ag-specific proliferation here is presented as Δ counts per minute. Proliferations of untreated and peptide-treated C6 cells in response to rhIL-2 (10 U/ml) alone were 10,272 and 42,315 cpm, respectively, when the proliferation was measured on day 3.

Results

Exposure of C6 cells to soluble peptide-induced minimal proliferation, apoptotic cell death, and nonresponsiveness in the surviving cells

To investigate the effects of Ag presentation by T cells, C6 cells were incubated with increasing concentrations of soluble H-Y Kk peptide in the absence or the presence of irradiated CBA female splenocytes (5 x 10^7/well) for 3 days. As shown in Figure 1A, T:T presentation induced only low level proliferation, amounting to approximately 20% of that observed in the presence of anti-IL-4 mAb (11B11) for 2 days. C6 cells (5 x 10^5/well) were incubated with medium or 100 nM peptide overnight, and the cells were washed and rested for another day in fresh medium containing 10 µg/ml of anti-Kα mAb (HB25). The peptide-untreated and peptide-treated C6 cells (1 x 10^7/well) were then restimulated with peptide-prepulsed CBA female splenocytes (5 x 10^7/well) for 3 days. C, Unresponsiveness correlated with a defect in IL-2 production. After 36 h of rechallenge cultures, supernatants from untreated (Medium) or peptide-treated C6 cells (Pep) were harvested and assayed for IL-2 production using CTLL-2 cells. CTLL-2 cells (3 x 10^5/well) were cultured with supernatants at a final concentration of 25% (v/v) in the presence of anti-IL-4 mAb (11B11) for 2 days. D, Unresponsiveness reflected anergy rather than further activation-induced cell death during the rechallenge culture. C6 cells (5 x 10^5/well) were incubated with medium or 100 nM peptide overnight, and the cells were washed and rested for another day in fresh medium containing 10 µg/ml of anti-Kα mAb (HB25). Then these peptide-untreated and peptide-treated C6 cells (1 x 10^5/well) were restimulated with peptide-prepulsed CBA female splenocytes (5 x 10^7/well) in the presence of exogenous rhIL-2 (10 U/ml) for 3 days. Ag-specific proliferation here is presented as Δ counts per minute. Proliferations of untreated and peptide-treated C6 cells in response to rhIL-2 (10 U/ml) alone were 10,272 and 42,315 cpm, respectively, when the proliferation was measured on day 3.
peak after exposure to 100 nM peptide, the same concentration that induced optimal proliferation. Further, the T cells could not be protected from apoptosis by the addition of exogenous rhIL-2, as shown in Table I, Expt. 2. To determine the fate of the 60% cells that survived after overnight culture with soluble peptide, the surviving cells were harvested, rested for 24 h in the presence of anti-K\textsuperscript{b} mAb to prevent further T:T presentation, and then rechallenged with peptide-pulsed spleen cells. As shown in Figure 1B, the surviving cells were profoundly hyporesponsive compared with those that had been cultured in medium alone. The reduced Ag reactivity could not be accounted for by TCR:CD3 modulation, because levels of IL-2 production, as shown in Figure 1C. To exclude the possibility that the surviving cells had already been programmed to die and were undergoing apoptosis in the rechallenge culture, rechallenge was conducted in the presence of exogenous IL-2. As shown in Figure 1D, the addition of IL-2 restored the level of proliferation of the hyporesponsive cells to that of peptide-pulsed spleen cells, although the dose response was shifted to the right. These data suggest that T:T Ag presentation induced both apoptosis and anergy in the IL-2-producing, CD8\textsuperscript{+}, C6 T cell clone.

To exclude the possibility that the hyporesponsiveness induced by culture with soluble peptide reflected premature T cell restimulation, the T cells were rested for 6 days before rechallenge. The T cells were equally unresponsive after the prolonged rest period, but proliferated in response to rhIL-2, indicating that the lack of response to Ag reflected anergy, and that the anergic state persisted for at least 1 wk in vitro (data not shown).

C6 cells express high levels of CD80 and CD86, and efficiently stimulate primary T cell responses.

It has been reported previously that activated human and murine T cells express B7 family molecules (39–41). In light of the findings that Ag presentation by C6 T cells to other members of the same T cell clone induced apoptosis and anergy, the phenotype of the T cells was examined. The most striking feature was the levels of CD80 and CD86, as shown in Figure 3. As can be seen, the levels of both B7 isosforms exceeded those detected in DCs grown from bone marrow cultures in the presence of GM-CSF. The level of CD86, in particular, substantially exceeded that on the DCs.

The ability of C6 T cells to provide costimulation was investigated by testing their capacity to act as accessory or APCs for three categories of primary T cell response: mitogen-stimulated T cell proliferation, an alloimmune mixed lymphocyte reaction involving CD8\textsuperscript{+} responder T cells, and primary Ag-specific proliferation using TCR-transgenic T cells as responder cells. In all cases the C6 cells were superior to splenic APCs, although less efficient than DCs (Fig. 4). For peptide presentation to the TCR-transgenic T cells, the CD4\textsuperscript{+} T cells were unable to induce any proliferation, suggesting that costimulation is required to initiate proliferation even in the face of such a high precursor frequency. The contribution of the B7 molecules expressed by the C6 cells to the Con A and the MLR responses was assessed by the addition of anti-CD80 plus anti-CD86 mAbs or of CTLA4Ig fusion protein to the cultures containing C6 cells. Both these approaches to inhibiting the delivery of costimulation almost completely abolished the proliferative responses supported or induced by C6 (data not shown).

Peptide-triggered cell death requires bidirectional T:T presentation, but is not mediated by veto-, TNF-\alpha receptor-, or Fas-dependent mechanisms.

The results of incubating C6 cells with soluble peptide were apparently incompatible with the manifest capacity of C6 cells to function as accessory or APCs for primary T cell responses and with the fact that C6 has the phenotypic credentials of specialized APC. In an attempt to resolve these conflicting data, the effects of
using peptide-pulsed C6 cells as APC for unpulsed C6 were examined. As presented in Figure 5A, this led to markedly increased proliferation that was greater than that induced by peptide-pulsed splenic adherent cells. No proliferation was induced by peptide-pulsed CD4\(^+\) T cells, suggesting that the C6 clone depends upon B7-mediated costimulation to proliferate. Furthermore, when C6 cells were exposed to peptide-pulsed C6 APC, no apoptosis was induced (data not shown). The major difference between this approach and the incubation of C6 with soluble peptide is that using peptide-pulsed C6 cells as APC ensures that the Ag presentation is unidirectional rather than bidirectional.

Several possible mechanisms for the inhibitory effects of T cell Ag presentation were tested. The first was that T cell Ag presentation led to death of the responding cell, resembling a veto-like phenomenon (42–44). The proliferation of C6 cells in response to unidirectional presentation makes a veto-like phenomenon highly unlikely.

Two additional mechanisms that could be responsible for inducing apoptosis as a result of T:T Ag presentation were signaling through Fas (45) or through the TNF-\(\alpha\) receptor (46). TNF-\(\alpha\)-mediated signaling was unlikely to have contributed, in that no TNF-\(\alpha\) was detected in the culture supernatants of C6 cells incubated with soluble peptide (<20 pg/ml; data not shown). In addition, although the presence of a rabbit anti-TNF-\(\alpha\) antiserum led to a slight reduction in the percentage of apoptotic cells following incubation with 100 nM peptide, this protection was less than that seen with a normal rabbit serum, and no protection was observed at the higher peptide dose (Table I, Expt. 3).

Moreover, no membrane-bound TNF-\(\alpha\) on C6 cells was observed using an anti-TNF-\(\alpha\) serum even after activation, although these Abs produced positive staining in some T cell clones, such as IF8 (Fig. 5, B and C), and inhibited rTNF-\(\alpha\) binding in the standard ELISA when they were used as blocking Abs (data not shown). Additional studies indicated that peptide-induced apoptosis of C6 cells was independent of the Fas-FasL pathway. First, C6 cells even after activation were completely negative for Fas expression as detected by the mAb Jo2, which could significantly stain CBA thymocytes (Fig. 5, D and E). Second, C6 cells also lacked expression of FasL, as detected by staining with Kay 10, an anti-FasL mAb (data not shown). Finally, Jo2 Abs did not rescue the cells from peptide-triggered death (data not shown).

An alternative mechanism of inducing apoptotic cell death that has been reported for mouse and human T cells results from ligation of cell surface MHC class I molecules (47, 48). This would be an attractive way to account for the effects of bidirectional T cell presentation, in that cognate T:T interactions would lead to simultaneous ligation of the TCR and peptide-occupied class I molecules. The effects of ligation K\(^\alpha\) molecules on C6 cells were tested by culturing the cells on plates coated with anti-K\(^\alpha\) Ab, with or without anti-CD3 Ab. The presence of anti-K\(^\alpha\) Ab did not lead to cell death on its own, nor did the anti-class I Ab increase the percentage of cells undergoing apoptosis in response to immobilized anti-CD3 Ab (Table II).

High level expression of CTLA-4 was induced on C6 cells after exposure to soluble peptide, but was not responsible for the induction of apoptosis and anergy

It is well established that the two receptors for B7 molecules, namely CD28 and CTLA-4, have contrasting functions (37, 49–52). CD28-mediated signals amplify IL-2 production by T cells by increasing IL-2 gene transcription and by increasing the stability of IL-2 mRNA (5, 53). In contrast, CTLA-4 signaling appears to negate the positive effects of CD28 signaling, possibly through the activation of CTLA-4-linked phosphatases (54). As a consequence, CTLA-4 ligation has been associated with both cell death (55) and the induction of T cell anergy (56). For these reasons, the possible involvement of CTLA-4 signaling in the inhibitory effects of T:T presentation were investigated.

First, the pattern of B7 molecule expression following addition of soluble peptide to C6 cells was characterized. As shown in Figure 6A, soluble peptide led to a reversal of the ratio of CD80 to CD86. Two weeks after the last Ag stimulation, at the time that T
results were reproduced when the T cells were stimulated with peptide-pulsed C6 cells or splenic APC (data not shown).

Given that CD80 has a slower dissociation rate from CTLA-4 than CD86 (57, 58), it was of interest to determine whether CTLA-4 expression was induced following exposure of the C6 cells to peptide. Little variation was seen in the expression of CD28; CTLA-4, in contrast, was undetectable on the resting cells, but was expressed at substantial levels on the cells exposed to 100 or 1000 nM peptide, peaking between days 2 and 3 after addition of peptide. Only very low level and transient expression of CTLA-4 was observed after incubation of C6 with peptide-pulsed C6 cells or splenocytes (data not shown).

The above data raised the possibility that some of the negative effects of T:T presentation might be accounted for by CTLA-4 signaling. This was tested by adding intact anti-CTLA-4 Ab or a Fab preparation to C6 cells cultured with soluble peptide. The addition of the anti-CTLA-4 Ab in either form conferred no protection from cell death, as shown in Table I, Expt. 4 and 5; similarly, the addition of anti-CTLA-4 with soluble peptide and during the rest period led to no protection form the induction of anergy (Fig. 7, A and B). The addition of anti-CTLA-4 mAb or Fab fragments did, however, augment the level of proliferation in response to soluble peptide, as shown in Figure 7, C and D. Moreover, the addition of anti-CTLA-4 mAb led to a >10-fold increase in Ag-specific proliferation in responsive C6 cells (Fig. 7, A and B).

Discussion

The results presented here demonstrate that incubation of a murine CD8\(^+\), IL-2-producing, T cell clone with specific peptide leads to nonresponsiveness due to the induction of apoptosis in a fraction of the cells and of anergy in the remainder. Investigation of the mechanisms underlying the induction of nonresponsiveness suggest that it reflects a novel mechanism of nonresponsiveness, in that it required bidirectional T:T Ag presentation and did not involve CTLA-4 ligation, and neither Fas or TNF-\(\alpha\) was instrumental in causing the cell death.

Apoptosis was consistently induced in approximately 40% of the T cells, and cell death occurred within 24 h of culture of the cells with soluble peptide (Table I, Expt. 1, and Fig. 2). This appeared to require bidirectional Ag presentation, in that no cell death was seen when C6 T cells were cultured with peptide-pulsed members of the clone (data not shown). Indeed, such unidirectional presentation led to a significant proliferative response (Fig. 5A). A variety of mechanisms could be implicated to account for the cell death observed. The simplest explanation would be that the C6 cells had cytolytic activity and caused fratricide (59, 60). This is unlikely to explain these results, in that C6 does not have lytic activity against H-2\(^b\) male target cells (29), and CTL are generally refractory to conventional cytotoxicity. Another possibility is that the peptide-pulsed cells engaged in suicide (61, 62), as originally proposed by Walden and Eisen (61) in a similar system. This would be consistent with the results presented here. Nonetheless, whether the nonresponsiveness resulted from bidirectional presen-
tation or ligation of the TCR by peptide:MHC complexes on the same cell surface, the critical event would appear to be the simultaneous presentation of and recognition of Ag by a single T cell (63). Any potential contribution to cell death due to the veto phenomenon (42–44), first described by Miller and colleagues (42), was ruled out because peptide-prepulsed C6 cells failed to lyse sister C6 cells, as assessed by the JAM test (64) (data not shown).

Several molecular interactions have been implicated in the in-
duction of apoptosis by T cells. For CD4\(^+\) T cells, Fas ligation is

**FIGURE 4.** B7-1 and B7-2 expressed on C6 cells are functional co-stimulatory molecules. A, Con A response. Purified CBA female CD4\(^+\) T cells (5 \(\times\) 10\(^4\)/well) were stimulated with the indicated concentrations of Con A in the presence of irradiated DCs (5 \(\times\) 10\(^4\)/well), irradiated C6 cells (5 \(\times\) 10\(^4\)/well), or irradiated female CBA splenic adherent cells (5 \(\times\) 10\(^5\)/well) for 3 days. These data are representative of two experiments. B, Allogeneic MLR. Purified BALB/c female CD8\(^+\) T cells (1 \(\times\) 10\(^5\)/well) were incubated with the indicated numbers of irradiated female CBA DCs, irradiated C6 cells, or irradiated CBA female splenic adherent cells for 3 days. These data are representative of three experiments. C, C6 cells present antigenic peptide to activate resting Ag-specific transgenic CD8\(^+\) T cells. Purified CD8\(^+\) T cells (5 \(\times\) 10\(^5\)/well) from TCR-transgenic mice were incubated with irradiated peptide-prepulsed C6 cells (5 \(\times\) 10\(^5\)/well), irradiated peptide-prepulsed CBA female splenic adherent cells (5 \(\times\) 10\(^5\)/well), or irradiated peptide-prepulsed purified CBA female CD4\(^+\) T cells (5 \(\times\) 10\(^5\)/well) for 3 days.
a major pathway of cell death (45), while for CD8 T cells, ligation of the TNF-α receptor may play a complementary role (46). Both these possibilities were investigated in the present studies. We were unable to detect Fas expression on C6 T cells either at rest or after exposure to soluble peptide (Fig. 5D), effectively excluding Fas-induced signals as being responsible for the induction of apoptosis. Similarly, we could not detect soluble or membrane-bound forms of TNF-α in the supernatants (20 pg/ml) or on the surface of activated C6 cells (Fig. 5B), nor was it possible to protect the T cells from apoptosis by the addition of anti-TNF-α Ab (Table I, Expt. 3). Thus, the well-defined signals that have been associated with T cell apoptosis did not appear to be applicable in this system. An alternative mechanism of inducing apoptotic cell death that has been reported for mouse (47) and human T cells (48) results from ligation of cell surface MHC class I molecules. This would be an attractive way to account for the effects of bidirectional T cell presentation, in that cognate T:T interactions would lead to simultaneous ligation of the TCR and peptide-occupied class I molecules. The effects of ligating Kk molecules on C6 cells were tested by culturing the cells on plates coated with anti-Kk Ab, with or without anti-CD3 Ab. The presence of anti-Kk Ab did not lead to cell death on its own, nor did the anti-class I Ab increase the percentage of cells undergoing apoptosis in response to immobilized anti-CD3 Ab (Table II).

Table II. Immobilized anti-CD3 mAb-triggered apoptosis in C6 cells was inhibited by coimmobilized anti-Kk mAb

<table>
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<th>Condition</th>
<th>0 µg/ml</th>
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<th>0.1 µg/ml</th>
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<td>6.0</td>
<td>16.0</td>
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C6 cells (1 × 10⁵/well) were cultured in 0.2 ml of 10% FCS RPMI 1640 medium containing IL-2 (10 U/ml) for 2 days in flat-bottomed 96-well plates previously coated overnight with the indicated concentration of anti-CD3 (145-2C11), anti-Kk (HB25), or both. Apoptosis was determined by PI staining and flow cytometry analysis. This experiment is representative for three times. Anti-H-2Ea (clone 14.4.4S) was used as an isotype-matched control antibody which failed to affect anti-CD3-triggered cell death.

Percent of apoptosis. ND, not determined.
Care was taken to exclude the possibility that the lack of proliferation seen upon restimulation of the peptide-treated cells was due to the induction of apoptosis upon rechallenge. No increase in apoptotic cells was seen in the rechallenge cultures (data not shown); furthermore, addition of IL-2 to the rechallenge cultures restored proliferation (Fig. 1D). The simplest explanation for the induction of anergy would be that it resulted from a lack of B7-mediated costimulation. This clearly was not applicable, in that the C6 cells expressed remarkably high levels of B7 molecules (Fig. 3, A and B).

**FIGURE 6.** A, Reciprocal expression of B7-1 and B7-2 on C6 cells after exposure to soluble peptide. Purified C6 cells (5 × 10^5/well), 14 days after their last stimulation, were incubated with 10, 100, or 1000 nM peptide in 24-well plates and analyzed for B7-1 and B7-2 expression before (day 0) or after (days 1–4) stimulation with peptide. Live C6 cells were enriched by low speed centrifugation to remove residual APCs before being incubated with peptide. B7 expression on C6 cells was determined by indirect immunofluorescence staining. At the indicated time (days) after incubation with peptide, C6 cells were isolated and stained with anti-B7-1 (1G10) or anti-B7-2 (GL-1) mAb followed by FITC-conjugated rabbit anti-rat IgG (Dako; thin and dotted line, respectively). Anti-CD4 mAb (YTS191) was used as an isotype-matched control (thick line). These data are representative of four experiments. B, Kinetic expression of CD28 and CTLA-4 on C6 cells after exposure to soluble peptide. After overnight incubation with peptide, C6 cells (5 × 10^5/well), 12 days after their last stimulation, were incubated with a range of peptide concentrations and were analyzed for CD28 and CTLA-4 expression at 24-h intervals. CD28 and CTLA-4 expressions were determined by indirect immunofluorescence staining with anti-CD28 (37.51) or anti-CTLA-4 (UC11-4F10–11) mAb followed by FITC-conjugated goat anti-rat IgG (Sera-Lab; thin and dotted lines, respectively). Anti-TCRγδ (GL-3) was used as an isotype-matched control (thick line). These data are representative of four experiments.
The levels of B7 molecules expressed by the C6 T cells were surprising, in that they exceeded those expressed by DCs cultured from bone marrow in the presence of GM-CSF (Fig. 3, C and D). As befits cells with these levels of costimulatory molecules, C6 cells were effective accessory cells for mitogen responses by highly purified resting CD4<sup>+</sup>T cells, were able to stimulate a MLR by allogeneic CD8<sup>+</sup>T cells, and were potent APC for unprimed CD8<sup>+</sup> TCR-transgenic T cells (Fig. 4). These observations rule out the possibility that any of these phenomena was due to a lack of costimulation; however, the altered pattern of expression of the two isoforms of B7 following exposure to Ag (Fig. 6A) raised the question of whether signaling through CTLA-4 could contribute to the induction of nonresponsiveness. Although both isoforms of B7 demonstrably interact with both of the counterreceptors, CD28 and CTLA-4, the CD80 molecule has a slower dissociation rate from CTLA-4, and CD86 has a higher affinity for CD28 (57, 58). Furthermore, recent data have raised the possibility that CTLA-4-mediated signals are instrumental in the induction of T cell anergy (56).

The relevance of CTLA-4 signaling to the consequences of T:T Ag presentation was further suggested by the observation that incubation of the C6 T cells with soluble peptide led to a dose-dependent increase in CTLA-4 expression (Fig. 6B). The levels of CTLA-4 expression induced by soluble peptide were much higher than those induced by incubation of C6 cells with peptide-pulsed spleen cells or C6 cells. Indeed, culture of C6 cells with soluble peptide in the presence of anti-CTLA-4 Ab or Fab doubled the level of proliferation observed (Fig. 7, C and D). Despite this, we were unable to protect the T cells from the induction of either apoptosis or anergy by adding intact or a Fab preparation of the anti-CTLA-4 Ab (Fig. 7, A and B).

These data illustrate the potent regulatory consequences of T:T Ag presentation. The nonresponsiveness that results appear to reflect a novel mechanism, in that it cannot be readily explained by currently identified pathways that lead to apoptosis or anergy in T cells. The interest in these phenomena is in their possible relevance to the functional consequences of B7 expression by T cells. Clearly, mouse T cells are unlikely to act as APC in vivo. This is certainly true for CD4<sup>+</sup> responder T cells, in that they do not express MHC class II molecules. However, the fate of T cells transferred into MHC-deficient mice suggests that low affinity cognate interactions with self-MHC molecules are responsible for the survival of T cells in vivo (65). This requires the maintenance of a fine balance between signaling to maintain survival and activating T cells with autoreactive potential. The regulatory effects of cognate T:T interactions observed here may represent an exaggerated form of events that occur in vivo and may serve to inhibit the
activation of autoreactive T cells and the regulation of T cell responses.

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References


