Brief Definitive Report

CD28–B7 Interactions Allow the Induction of CD8$^+$ Cytotoxic T Lymphocytes in the Absence of Exogenous Help

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Summary

The activation requirements for the generation of CD8$^+$ cytotoxic T cells (CTL) are poorly understood. Here we demonstrate that in the absence of exogenous help, a CD28–B7 interaction is necessary and sufficient for generation of class I major histocompatibility complex–specific CTL. Costimulation is required only during the inductive phase of the response, and not during the effector phase. Transfection of the CD28 counter receptor, B7, into nonstimulatory P815 cells confers the ability to elicit P815-specific CTL, and this response can be inhibited by anti-CD28 Fab or by the chimeric B7-binding protein CTLA4Ig. Anti-CD28 monoclonal antibody (mAb) can provide a costimulatory signal to CD8$^+$ T cells when the costimulatory capacity of splenic stimulators is destroyed by chemical fixation. CD28-mediated signaling provokes the release of interleukin 2 (IL-2) from the CD8$^+$ CTL precursors, as anti-CD28 mAb could be substituted for by the addition of IL-2, and an anti-IL-2 mAb can block the generation of anti-CD28–induced CTL. CD4$^+$ cells are not involved in the costimulatory response in the systems examined. We conclude that CD8$^+$ T cell activation requires two signals: an antigen-specific signal mediated by the T cell receptor, and an additional antigen nonspecific signal provided via a CD28–B7 interaction.

Naive CD4$^+$ T cells and Th1 clones require two signals for full activation: an antigen-specific signal via the antigen receptor and a second signal via an antigen nonspecific costimulatory receptor (1–3). In the absence of the costimulatory signal, naive T cells exhibit suboptimal proliferation and cytokine secretion (4). It has been demonstrated recently that the costimulatory signal can be mediated by an interaction between the T cell surface molecule CD28 and its ligand B7 (5–8).

The activation requirements for naive CD8$^+$ cells in the generation of an MHC class I–restricted response are less well understood. It has been suggested that generation of CD8$^+$ CTL requires interaction with class II–restricted Th cells (9, 10), and that when TCR affinity is low, CD4$^+$-mediated T cell help is required in vivo (11). Recent evidence indicates that although exogenous help from CD4$^+$ Th in the form of IL-2 may enhance CTL responses by expansion of activated precursors, this exogenous help is not always obligatory. In vitro studies have demonstrated that CD8$^+$ cells can be stimulated to proliferate and generate CTL responses to alloantigens in the absence of CD4$^+$ Th cells (12–16). Moreover, it has been demonstrated that mice in which CD4$^+$ cells have been eliminated by disruption of the CD4 gene by homologous recombination can nonetheless mount an effective CTL response to viral challenge (17).

Although it has been previously suggested that two signals might be required for an effective CTL response (18), there is at present little direct data concerning the role of costimulatory signals. The likelihood that costimulation is involved was suggested by the observation that the addition of accessory cells, in the form of class II$^+$ cells, adherent cells, or macrophage cell lines was necessary for a primary CD8$^+$ response in the absence of CD4$^+$ cells (12, 13). In addition, cloned macrophage lines (15) and dendritic cells (14) can directly activate CD8$^+$ T cells. Whereas B7 has been demonstrated to be present on macrophages and dendritic cells (19, 20), its direct involvement in providing costimulatory signals to CD8$^+$ T cells has not been demonstrated. Recently, it has been shown that B7 transfected-cells elicit potent protective immune responses even in the absence of CD4$^+$ T cells (21, 22).

Here we examine the role that CD28 and its counter receptor, B7, play in the generation of primary responses in two class I–specific allogeneic responses in vitro: H-2^b anti-H-2$^{bm\text{-}1}$ and H-2^k or H-2^b anti-H-2^d. We demonstrate that cytotoxic responses to nonstimulatory P815 mastocytoma cells can be induced by B7-transfected P815 cells, and that the costimulatory signal can be blocked by Fab fragments of anti-CD28 antibody or by the chimeric B7 ligand CTLA4Ig. CD8$^+$ T cells can mature to CTL in the absence of CD4$^+$ helper cells. Splenic stimulator cells express an essential fixation-labile signal that can be replaced by anti-CD28 antibody. We

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also demonstrate that the costimulatory effect is most likely a function of IL-2 secretion by the responding cells. Finally, we demonstrate that whereas CD28–B7 interactions are required for the induction phase of the response, they are not necessary during the effector phase.

Materials and Methods

Cells and Cell Lines. C3H/HeN (H-2^k) mice were obtained from Charles River Laboratories (Wilmington, MA). C57Bl/6 (H-2^b) and B6.CH-2^{bm-1} (H-2^{bm-1}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). LN T cells were prepared by treatment of total LN cells with anti-MHC class II mAb containing supernatants and a mixture of rabbit (Cedarlane, Hornby, Ontario, Canada) and guinea pig (Gibco, Grand Island, NY) complement. Negative selection for residual class II$^+$ B cells was accomplished by panning on anti-mouse Ig coated plates. Occasionally, LN cells were passed over nylon wool before complement lysis. Both procedures resulted in >90% T cells. CD8$^+$ T cells were prepared from LN T cells by incubation with anti-CD4 mAb followed by complement lysis or panning on anti-rat Ig coated plates. These populations were enriched to >85% CD8$^+$. The remaining cells were <0.5% CD4$^+$. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (ECDI)–fixed splenocytes were prepared in 75 mM ECDI as described (23). MC201A5, p815 transfected with the mouse B7 gene (B7-P815), was provided by Dr. L. Lanier (DNAX, Palo Alto, CA) (24). Nontransfected P815 and B7-P815 were cultured in 10% FCS-supplemented RPMI 1640 containing 2-ME, gentamycin, pyruvate, glutamine, and Hepes (complete medium). Transfectants were checked periodically for B7 expression during the course of these experiments by flow cytometry using CTLA4Ig.

Reagents. CTLA4Ig (25) was a gift of Dr. P. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Anti-IL-2 mAb was used as a culture supernatant from S4B6 hybridoma cells, or purchased from PharMingen (San Diego, CA). Anti-CD28 mAb (26) was purified from ascites fluid from the hybridoma 37.51. Fab fragments of anti-CD28 and F560.31, a hamster IgG control antibody, were prepared by proteolysis with immobilized papain (Pierce Chemical Co., Rockford, IL), and undigested antibody removed by adsorption on protein A-Sepharose.

Generation of Cytotoxic Cells. LN or CD8$^+$ T cells at 5 × 10^6 per ml were cocultured with stimulator cells in complete medium at 37°C in 5% CO_2 for 5 d. Stimulator cells were irradiated splenocytes at a 2:1 stimulator/responder ratio, ECDI-fixed splenocytes at a 3–5:1 ratio, or irradiated p815 or B7-P815 at a 1:5 ratio. Before some assays, viable cells were purified by centrifugation over Histopaque (δ = 1.119; Sigma, St. Louis, MO), in which case cytotoxic capacity was reported as E/T ratios. When cultures were tested for cytotoxicity without repurifying effector cells, cytotoxicity was reported as input to target ratios. Effector T cells were not repurified when cultures yielded few viable cells, such as mixed lymphocyte culture (MLC) with ECDI-fixed targets, which had an average yield of 2% viable cells over five experiments. Cytotoxicity assay targets were either splenic LPS blasts or transformed cell lines. Target blasts were fractionated over Histopaque (δ = 1.077; Sigma), then labeled by incubation with 300 mCi ^{51}Cr for 90 min. Transformed cell line targets were labeled directly without activation for 60 min. Dilutions of effector cells were prepared in complete medium, and ^{51}Cr-labeled cells were added at 10^4 per well for transformed cell line targets, and 2 × 10^4 per well for LPS blast targets to a final volume of 200 μl per well. Assays were incubated at 37°C for 4 h. 100 μl of culture supernatant from each well was removed and processed for the calculation of specific ^{51}Cr release.

Table 1. *Coculture with B7-transfected, MHC Class I Allogeneic Stimulators Specifically Enriches for CD8$^+$ T Cells*

	Cells recovered	Percent CD4/CD8	Yield CD4	Yield CD8
	× 10^6	%	× 10^6	
Input LN T	13	68/26	8.8	3.4
LN T cocultured with				
P815	5.5	66/22	3.6	1.4
B7-P815	9.1	30/63	2.7	5.7

LN T cells from C3H/HeN mice were cocultured with P815 or B7-P815 as described. Viable cells were isolated. Numbers of CD4$^+$ and CD8$^+$ cells were determined using two-color flow cytometric analyses. The percentages of CD4$^+$ and CD8$^+$ T cells in the input population were determined on day 0 of culture.

Results and Discussion

To directly assess the role of B7 in the cytotoxic response, we compared the capacity of P815 mastocytoma cells (H-2^d, MHC class II$^-$) and B7-transfected P815 cells to elicit specific CTL from C3H T cells (H-2^k) in a MLC. CD8$^+$ cells selectively proliferated in MLC with B7-P815, whereas P815 MLC yielded many fewer cells and a CD4/CD8 ratio similar to the input population (Table 1). Consistent with the above result, little anti-P815 cytotoxic activity was elicited in control P815 MLC, whereas B7-P815 stimulators elicited

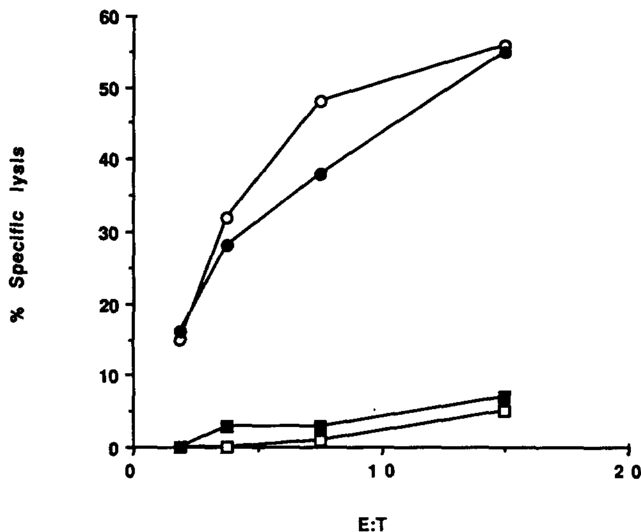

Figure 1. B7 expression on stimulator cells allows induction of class I anti-allogeneic cytotoxicity, but is not required for effector function. C3H LN T cells were cocultured with P815 or B7-P815 as described in Materials and Methods. Viable effector T cells were purified from MLC and tested for cytotoxicity against both P815 and B7-P815. (■, □) cytotoxic activity of T cells stimulated with P815 cells; (●, ○,) those stimulated with B7-P815. (■, ●) B7-P815 targets; (□, ○) P815 targets. This experiment has been performed six times with similar results.

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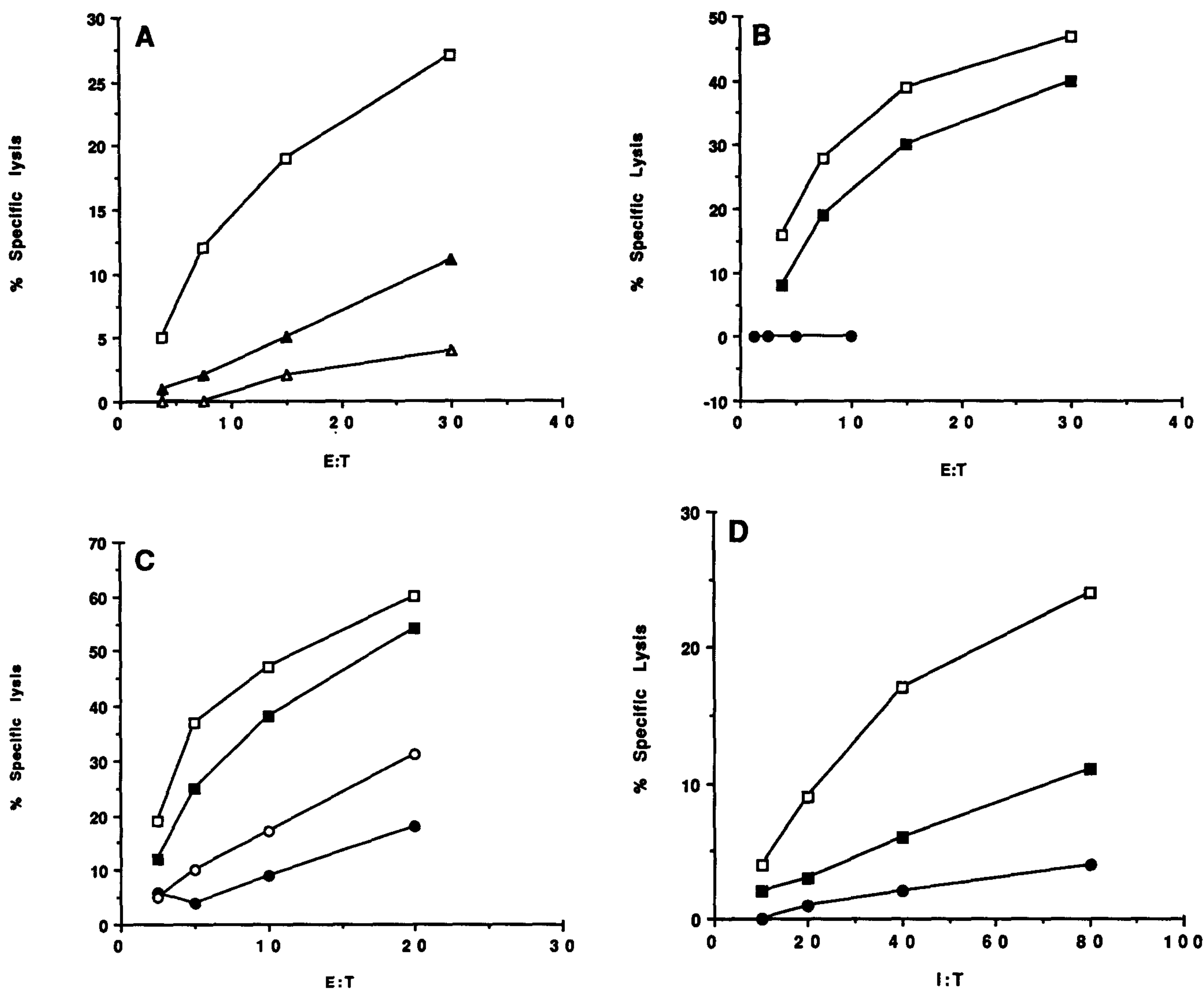

Figure 2. Generation of CTL requires a CD28–B7 interaction, or IL-2. (*A*) CTLA4Ig blocks the induction of cytotoxicity against murine B7-transfected P815. Viable C3H-derived effector T cells were purified from MLC. (□) Positive control cytotoxicity generated by C3H T cells in MLC with B7-P815. CTLA4Ig was included in MLC at 5 μg/ml (▲) and 25 μg/ml (△). Control culture cytotoxicity against self-blasts was 7% at an E/T of 30. (*B*) Anti-CD28 Fab fragment blocks the induction of cytotoxicity against murine B7-transfected P815 stimulators. (□) Control cytotoxicity generated by C3H T cells in MLC with B7-P815. Both the control F560.31 Fab (■) and anti-CD28 Fab (●) were included in MLC at 50 μg/ml. MLC containing anti-CD28 Fab yielded only enough viable cells upon repurification to perform titrations to an E/T of 10. Control culture cytotoxicity against EL-4 targets was 5% at an E/T of 15. (*C*) Anti-IL-2 mAb blocks the induction of CTL against murine B7-transfected P815. (□) Control cytotoxicity generated by C3H T cells in MLC with B7-P815. Anti-IL-2 supernatant (S4B6) at 10% (●) and 5% (○) in MLC. 25% F560.31 supernatant was used as a control (■). All targets were P815. Control culture cytotoxicity against third-party EL-4 cells was 15% at an E/T of 20. (*D*) Purified CD8$^+$ T cells can generate CTL in MLC with B7-P815. C57Bl/6 CD8$^+$ T cells were cocultured with B7-P815 (□), B7-P815 plus 70 μg/ml anti-CD28 Fab fragments (■), or B7-P815 plus 20 μg/ml purified anti-IL-2 (●), then were tested for cytotoxicity against P815 targets. Anti-CD28 Fab was not nonspecifically reducing responses in these cultures, as the inclusion of IL-2 in Fab containing cultures restored cytotoxicity to control levels in related experiments (data not shown). (*x axis*) MLC input to assay target ratios (*I:T*). MLC with P815 as stimulators generated no specific cytotoxicity against P815 targets at an I/T of 100, similarly to the data presented in Figure 1.

a potent CTL response (Fig. 1). This response was specific, since there was no significant cytolysis of L cells (H-2^k), B7-transfected L cells, or EL-4 (H-2^b) cells (data not shown). CTL from the B7-P815 cultures were equally effective in lysing B7-P815 and P815 (Fig. 1), confirming previous data which showed that the expression of B7 by the target cell is not essential for effector function of the CTL (24).

Blocking experiments also demonstrated the importance of CD28–B7 interactions in the generation of CTL activity. It has been demonstrated that CTLA4Ig, a chimeric protein composed of the extracellular domain of CTLA-4 fused to human IgG Cγ1 binds to the CD28 ligand B7 with high affinity (25). We assessed the effect of CTLA4Ig on the response of T cells to B7-P815 stimulators. As shown in Fig.

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2 *A*, CTLA4Ig effectively blocked the generation of specific CTL in a dose-dependent manner. Similarly, blockade of CD28 on the responding T cells with nonstimulatory Fab fragments of anti-CD28 antibody also inhibited the generation of CTL (Fig. 2 *B*).

Since it has been demonstrated that a major effect of CD28 costimulation on $CD4^+$ Th1 cells is to enhance the secretion of IL-2, we sought to determine the role of IL-2 in the CTL costimulatory response. The addition of anti-IL-2 during the induction phase prevents the generation of CTL to B7-P815 (Fig. 2 *C*). Direct assays of IL-2 in MLC supernatants indicated the presence of only very low levels, perhaps as a consequence of low production and rapid consumption by the responding $CD8^+$ cells (13).

Although it seemed unlikely that $CD4^+$ cells play a role in these CTL responses, as there was selective proliferation of $CD8^+$ (Table 1), the possibility of involvement of $CD4^+$ T cell help could not be ruled out. Presentation of allogeneic class I molecule–derived peptides could have occurred by residual APC in the responder T cell preparation, or as a result of upregulation of class II on P815 by cytokines secreted by activated T cells (27). Therefore, we examined the ability of purified $CD8^+$ T cells to generate CTL in MLC with P815 and B7-P815 stimulators. As shown in Fig. 2 *D*, purified $CD8^+$ T cells were capable of potent cytotoxic activity after coculture with B7-P815 but not P815. Similarly to unfractionated T cells, anti-CD28 Fab fragments and anti-IL-2 antibodies inhibited CTL generation.

Whereas these results clearly demonstrate that B7 transfection renders p815 capable of inducing antigen-specific CTL in vitro, the contribution of B7/CD28-mediated adhesion versus signaling was unclear. It has been demonstrated that chemical fixation destroys the ability of antigen-pulsed APC to stimulate IL-2 production and proliferation of $CD4^+$ T cells (23). This stimulation can be restored by the addition of allogeneic B cells or anti-CD28, indicating that fixation destroys costimulatory activity of the APC, while preserving the antigen signal (1, 3). This data also indicates that ECDI-fixed APC are capable of cognate interactions with T cells, suggesting that necessary adhesion interactions (28) are not significantly affected by ECDI treatment. We therefore tested the signaling versus adhesion component of the CD28 interaction using ECDI-fixed stimulator cells. The CTL response of C57Bl/6 T cells to $H\text{-}2^{bm\text{-}1}$ splenocytes can be blocked by CTLA4Ig, indicating that a B7 interaction is necessary for induction of CTL (Fig. 3 *A*). As shown in Fig. 3 *B*, untreated $H\text{-}2^{bm\text{-}1}$ splenocytes also elicit $H\text{-}2^{bm\text{-}1}$-specific CTL from purified $CD8^+$ T cells. MLC containing ECDI-fixed $H\text{-}2^{bm\text{-}1}$ stimulator cells yielded few viable cells after 5 d (average 2% of control unfixed stimulators in five experiments), and cultures did not yield cells capable of target cell lysis. Inclusion of anti-CD28 mAb in an MLC with ECDI-fixed $H\text{-}2^{bm\text{-}1}$ splenocyte stimulators resulted in the generation of antigen-specific CTL, confirming that ECDI destroys the costimulatory signal but not the specific antigen signal. Since soluble anti-CD28 contributes to the induction of CTL, but not to adhesion, the role of CD28 in these experiments is to provide a costimulatory signal rather than to stabilize the T cell–APC interaction.

The addition of 50 U/ml of IL-2 to the induction cultures with ECDI-fixed stimulators also restored the generation of specific CTL (Fig. 3 *C*). Cytotoxic activity was more potent in cultures containing exogenous IL-2, indicating that IL-2 is limiting in control cultures. The generation of CTL in MLC with fixed stimulators and anti-CD28 could be blocked by the presence of anti-IL-2 mAb (Fig. 3 *D*). This is consistent with the data in Fig. 2 *C*, and suggests that CD28 signaling affects IL-2 secretion from $CD8^+$ T cells. Proliferation and cytotoxic capacity of antigen and IL-2–stimulated cytotoxic cells is consistent with the differentiation and maturation scheme whereby activation of the TCR leads to the onset of an IL-2–responsive state that in turn results in mature cytotoxic cells. It is unknown if IL-2 is the only cytokine required, or if IL-2 signaling results in the secretion of other factors necessary for the complete maturation of cytotoxic potential (29).

Cloned CTL exhibit an ability to kill targets in the absence of costimulation, meanwhile becoming anergic in terms of IL-2 production (30). This split tolerance suggests that the signaling pathways for cytotoxicity and IL-2 secretion are distinct in terminally differentiated cells. This is confirmed by the results published elsewhere (24) and herein (Fig. 1) indicating that activated CTL no longer require a CD28 interaction to kill once they have matured. This also suggests that naive T cells require a CD28–B7 interaction to induce differentiation to a mature cytolytic phenotype, and that this differentiative event leads to the distinct signaling pathways for proliferation and cytolysis. Interestingly, a CD28–B7 cell surface interaction was found to be necessary in humans for CD3-redirected lysis by small resting T cells bearing the $CD45RO^+$ marker for memory cells (24). Memory cells retain their cytolytic phenotype as redirected lysis occurs within 4 h. This is further evidence of distinct signaling pathways for lysis and proliferation in mature cells. However, unlike activated CTL, memory cells require the presence of B7 on the surface of target cells. Memory cells may therefore represent a final differentiation of T cells, such that lysis of targets is again under the control of the tissue-specific regulation of a concomitant CD28–B7 interaction.

Recently, we and others (21, 22) have demonstrated that B7 transfection of nonimmunogenic tumors renders them susceptible to rejection. Rejection of B7-transfected tumors results in antigen-specific protection from subsequent tumor challenge. In addition, abrogation of $CD4^+$ T cells in the tumor hosts had no effect on tumor rejection in the primary responses. Therefore, it is likely that $CD8^+$ T cells can directly mount cytolytic responses in the absence of $CD4^+$ Th cells if precursor $CD8^+$ T cells encounter antigen and B7 on stimulator cells in vivo, as described herein.

Taken together, the results presented here indicate that a CD28–B7 signaling event is necessary for the generation of CTL in the absence of exogenous help. The CD28–B7 interaction results in the secretion of IL-2 by $CD8^+$ T cells, which allows for the proliferation of antigen-specific CTL.

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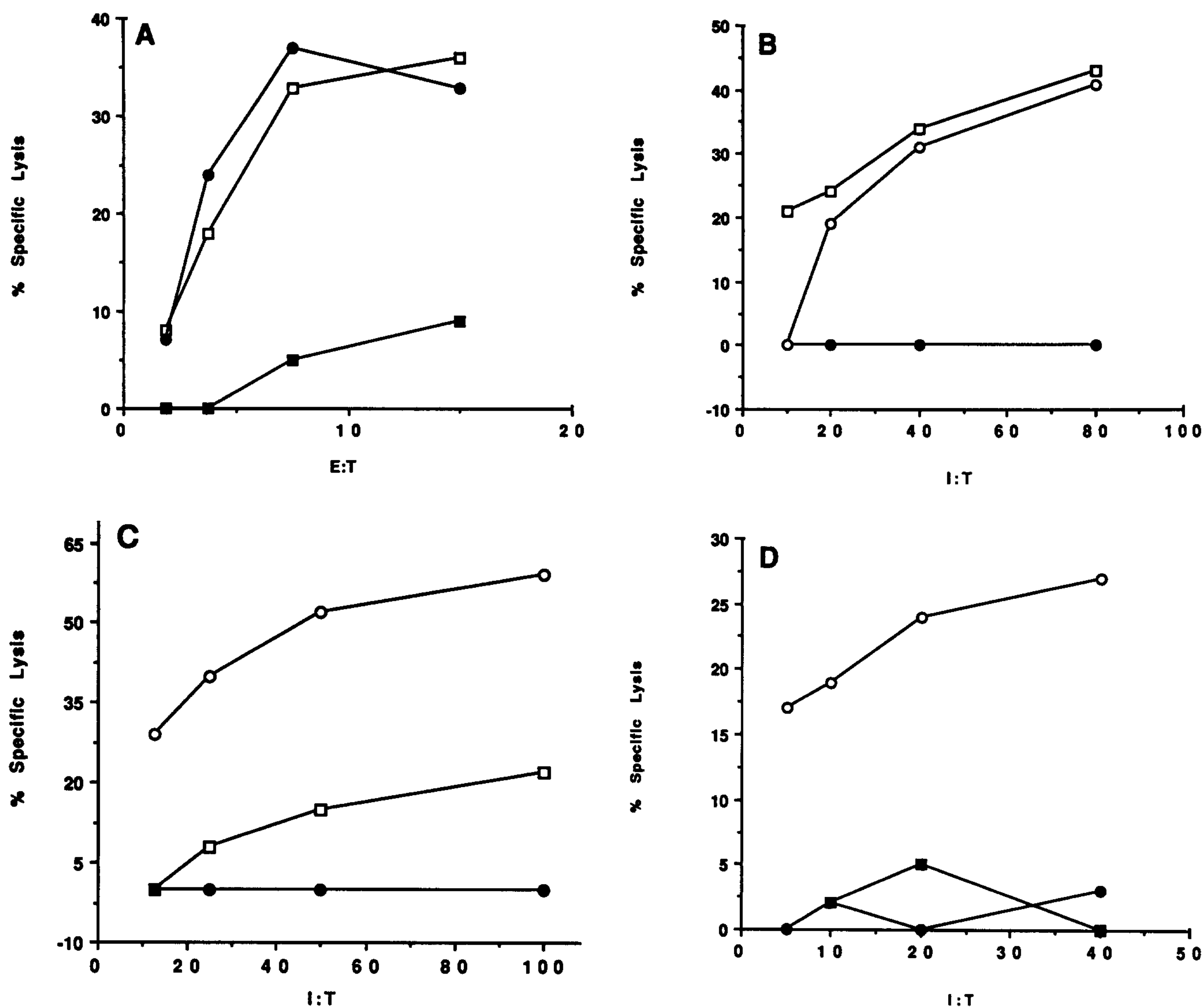

Figure 3. A fixation-labile signal is necessary for induction of CTL against H-2^{bm-1} and can be substituted for by anti-CD28 or IL-2. (*A*) CTLA4Ig blocks the induction of cytotoxicity by C57Bl/6 LN T cells against H-2^{bm-1} stimulators. C57Bl/6 LN T cells were cocultured with H-2^{bm-1} stimulators in complete medium (□), with 10 μg/ml CTLA4Ig (■) or 0.1 μg/ml CTLA4Ig (●). Viable effector T cells were purified. Targets were H-2^{bm-1} splenic blasts. No cytotoxicity was observed against self-blasts (data not shown). (*B*) Anti-CD28 mAb at 10 μg/ml restores the induction of specific cytotoxicity by C57Bl/6 $CD8^+$ T cells to ECDI-treated stimulator H-2^{bm-1} cells. $CD8^+$ C57Bl/6 T cells were cocultured with H-2^{bm-1} stimulators (□), with ECDI-fixed stimulators (●), or with ECDI-fixed H-2^{bm-1} stimulators and 10 μg/ml anti-CD28 mAb (○). Targets were H-2^{bm-1} splenic blasts. (*x-axis*) MLC input to assay target ratios (*I:T*). Cytotoxicity against third-party BALB/c and self-splenic targets at an I/T of 80 were 9 and 5%, respectively. This experiment has been performed six times with similar results. (*C*) IL-2 induces H-2^{bm-1}-specific CTL against ECDI-fixed stimulators. C57Bl/6 $CD8^+$ T cells were cocultured with H-2^{bm-1} stimulators (□), with ECDI-fixed stimulators (●), or ECDI-fixed H-2^{bm-1} stimulators and 50 U/ml IL-2 (○). Targets were H-2^{bm-1} splenic blasts. Positive control cultures exhibited no specific cytotoxicity against self-blasts at an I/T of 100. In cultures with IL-2, 2% cytotoxicity was seen to self-targets at an I/T ratio of 100. This experiment has been performed three times with similar results. (*D*) Anti-CD28–mediated induction of CTL to fixed H-2^{bm-1} stimulators can be blocked by anti-IL-2 mAb. C57Bl/6 $CD8^+$ T cells were cocultured with ECDI-fixed stimulators (■); ECDI-fixed H-2^{bm-1} stimulators and 10 μg/ml anti-CD28 mAb (○), or ECDI-fixed stimulators, 10 μg/ml anti-CD28, and 10% anti-IL-2 supernatant (●). Cytotoxicity against self- and third-party targets was $<2\%$ at an I/T of 40 for all groups. This experiment has been performed three times with similar results.

TCR stimulation and IL-2 are the minimum requirements for the generation of cytotoxic T cells in vitro. $CD4^+$ Th cells are not necessary, but may serve to amplify the response when activated to secrete IL-2.

This work was supported in part by a grant from the Irvington Institute for Medical Research (F. A. Harding) and National Institutes of Health grant CA-40041 (J. P. Allison).

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Received for publication 20 October 1992 and in revised form 15 March 1993.

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