Influence of Antigen Dose and Costimulation on the Primary Response of CD8+ T Cells in Vitro

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Summary

The influence of costimulation on the primary response of CD8+ T cells to class I alloantigens was studied with the aid of a T cell receptor transgenic model and defined peptides as antigen. With small doses of antigen, the proliferative response of CD8+ cells was high early in culture but was of brief duration and declined to low levels by day 4; this abbreviated response was associated with limited production of interleukin 2 (IL-2) and was strongly dependent upon costimulation via CD8–major histocompatibility complex class I and CD28–B7 interactions. The response to large doses of antigen was quite different in two respects. First, large doses of antigen inhibited the early (day 3) proliferative response but caused a marked elevation of the response late in culture (day 5); these altered kinetics were associated with increased production of IL-2. Second, the initial proliferative response to large doses of antigen did not require costimulation: indeed, blocking costimulation with CTLA4Ig or anti-CD8 monoclonal antibody enhanced the early proliferative response. However, blocking costimulation impaired IL-2 production and prevented the late proliferative response. These findings indicate that the requirement for costimulation of T cells can be partly overcome by increasing the dose of antigen to a high level. However, costimulation plays a key role in prolonging the response, presumably by triggering strong and sustained production of IL-2.

Primary responses of T cells are directed to peptide fragments of antigen bound to MHC molecules on APCs (1, 2). T cells recognize peptide–MHC complexes via α/β TCR molecules in conjunction with CD8 and CD4 coreceptors. By binding to MHC class I and class II molecules, respectively, CD8 and CD4 coreceptors promote TCR contact with peptide–MHC complexes and thereby augment the avidity of T–APC interaction. CD8 and CD4 molecules also enhance signaling by focusing tyrosine kinases such as p56^{lck} in the vicinity of TCR–CD3 complexes (3).

In addition to displaying peptide–MHC complexes, APCs express a variety of costimulatory molecules (1–4). These molecules interact with complementary molecules on T cells and deliver "second signals" for T cell activation. Particular attention has been focused on costimulation delivered via CD28–B7 interaction (5, 6). T cell CD28 molecules bind to either B7-1 or B7-2 molecules on APCs and are thought to transduce unique signals that stimulate T cell production of growth-promoting cytokines such as IL-2.

Although the notion that stimulation of unprimed T cells requires two qualitatively different signals has achieved wide acceptance, it is clear that costimulation is not unique to CD28–B7 interaction. Thus, several other molecules on

APCs, including intercellular adhesion molecule 1 (ICAM-1)[1] and heat-stable antigen (HSA), can exert quite potent costimulatory function (for review see reference 7). The implication therefore is that T–APC interaction is highly complex and involves multiple interactions between complementary sets of molecules on T cells and APCs. A priori, each set of molecules could induce unique patterns of signal transduction, the combined action of these different signals being required for optimal T cell stimulation. Alternatively, costimulatory molecules may function largely (or partly) as adhesion molecules and act by enhancing the avidity of T–APC interaction, thereby promoting optimal cross-linking of TCR–CD3 complexes (8).

In considering these two possibilities, it is important to know the minimal requirements for stimulating unprimed T cells. The literature on this topic is confusing. Thus, studies with CD28 knockout mice and blocking B7 function with CTLA4Ig indicate that CD28–B7 interaction is highly important in some situations (9) but not in others

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[1]*Abbreviations used in this paper:* DC, dendritic cell; HSA, heat-stable antigen; ICAM-1, intercellular adhesion molecule 1; MFI, mean fluorescence intensity; OGDH, 2-oxoglutarate dehydrogenase; PI, propidium iodide.

(10, 11). Likewise, the requirement for CD4 or CD8 coreceptors in primary responses is not an invariable finding (12, 13).

The simplest resolution to this problem is that the requirement for costimulation varies according to the affinity of TCR–peptide–MHC interaction: low affinity interactions are heavily dependent on signal boosting from coreceptors and costimulatory molecules, whereas high affinity interactions are not. In line with this idea, we present evidence that early primary responses of CD8$^+$ T cells to alloclass I–peptide complexes are only dependent on costimulation via CD8–class I and CD28–B7 interactions when the dose of antigen is low. In fact, with very strong TCR–peptide–MHC interaction, costimulation is inhibitory and causes a marked reduction of T cell responses. Paradoxically, however, the inhibitory effect of costimulation is transient and is followed by marked augmentation of the response later in culture.

Materials and Methods

Mice.   2C TCR transgenic mice, obtained from Dr. D. Loh (14), and B10.D2/NSnJ mice were bred and maintained at The Scripps Research Institute.

Cell Line and mAbs.   The following mAbs were used: 3.168 (anti-CD8) (15), RL172 (anti-CD4) (15), J11d (anti-HSA) (15), 28-16-8s (anti-I-A^b) (15), FD441.8 (anti-LFA-1) (15), and 30-5-7 (anti-L^d) (16). The RMA-S.L^d cell line and the anti-clonotypic 1B2 mAb were kindly provided by Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA) (16, 17). Purified CTLA4Ig fusion protein was a gift of P. Linsley (18).

Peptides.   Peptides were provided by Dr. A. Brunmark from R.W. Johnson Pharmaceutical Research Institute (La Jolla, CA) and were synthesized on a 431 A synthesizer (Applied Biosystems, Inc., Foster City, CA) and were purified with C18 reverse-phase HPLC. The concentrations of peptides were determined on the basis of their molecular weights.

Media.   For measuring proliferative responses, RPMI 1640 was supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 5% NCTC 109, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics (19).

Induction and Stabilization of Ld Expression on RMA-S.Ld Cells.   RMA-S.L^d cells precultured at 25°C overnight were incubated with peptides for 8–16 h at 25°C (20). Induction of L^d expression was then measured by staining the cells with anti-L^d mAb followed by a FITC-labeled second Ab (see flow cytometric analysis). For the stabilization assay, RMA-S.L^d cells preincubated with peptides at 25°C were cultured at 37°C for an additional 6 h and then stained for surface L^d expression. The mean fluorescence intensity (MFI) of staining was determined by FACS® analysis. The MFIs of L^d expression for RMA-S.L^d cells cultured at 37°C and at 25°C in the absence of peptides were 4 and 29, respectively.

Purification of CD8$^+$ and CD8$^-$ 2C Cells.   For cell purification (15), 2C LN cells were first treated with a cocktail of mAbs (anti-CD4, anti-HSA, anti-I-A^b) plus complement (C) for 45 min at 37°C. The surviving cells were further separated into CD8$^+$ and CD8$^-$ (CD4$^-$) cells by panning at 4°C for 60–90 min on petri dishes coated with anti-CD8 mAb. Nonattached cells were eluted and treated with anti-CD8 mAb and C to obtain CD8$^-$ 1B2$^+$ 2C cells. The attached (CD8$^+$) 1B2$^+$ 2C cells were recovered by incubation at 37°C for 5 min followed by vigorous pipetting.

Proliferation Assay.   Purified populations of CD8$^+$ or CD8$^-$ 2C cells were cultured with irradiated stimulator cells in 200-μl wells

(15). RMA-S.L^d stimulators were cultured overnight at 25°C in flasks, exposed to 3,000 cGy, washed, incubated at 5×10^5 cells per ml with peptides overnight at 25°C and then, without washing, plated out in 200-μl wells with the responder cells. For B10.D2 spleen or dendritic cells (DCs), these cells were exposed to 2,000 cGy, washed, incubated at 5×10^6 cells per ml with peptides at 25°C for 2 h, and then plated out with the responder cells without washing. Unless stated otherwise, responder cells were used at 2.5×10^4 cells per well; the dose of stimulator cells varied but was generally 5×10^4 cells per well for RMA-S.L^d cells, 5×10^5 cells per well for spleen cells, and $5–10 \times 10^4$ cells per well for DCs. DCs were purified according to established methods (19). To measure T cell proliferation, cultures were pulsed with 1 μCi of [^{3}H]thymidine 8 h before harvest. The data in the tables and figures refer to the mean of triplicate cultures; SDs were generally within 5–15% of the mean.

Generation of CTLs and CTL Assays.   By use of 2 ml volumes and 24-well plates, 5×10^5 CD8$^+$ 2C cells were cultured for 4 d with 5×10^5 RMA-S.L^d cells irradiated with 3,000 cGy and pretreated with peptides; peptides (p2Ca or QL9) were present during the culture at a concentration of 10 μM. To prepare targets, RMA-S.L^d cells were labeled with ^{51}Cr (100 μCi per $1–2 \times 10^6$ cells) at 37°C for 90 min in the presence or absence of peptides. After labeling, the cells were thoroughly washed and resuspended in medium with or without peptides. Specific ^{51}Cr release was calculated according to established procedures (15).

IL-2 Production.   IL-2 production was measured by using an IL-2–dependent cell line, CTLL (15). At the times indicated, 50 μl of culture supernatant was added to 5,000 CTLL cells for 24 h; 1 μCi [^{3}H]TdR was added 16 h before harvest. The data in the figures are expressed as the mean of triplicate cultures. IL-2 activity in the supernatants was not titrated routinely, although random titrations of supernatants showed a direct correlation with CTLL cpm below 80,000 cpm. Parallel titrations with rIL-2 showed that 20–30 U of IL-2/ml were required to reach 80,000 cpm with the CTLL line under the conditions used.

Flow Cytometric Analysis.   For analysis of surface expression of L^d on RMA-S.L^d cells, 5×10^5 cells were incubated with anti-L^d mAb for 30 min on ice, washed in PBS buffer with 2.5% horse serum and 0.1% of sodium azide, and then stained with FITC-conjugated goat F(ab)′$_2$ anti–mouse Fcγ antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). TCR expression on CD8$^+$ 2C cells was measured with FITC-conjugated 1B2 mAb. PE-conjugated anti-CD8 mAb was purchased from GIBCO BRL (Gaithersburg, MD). Dead cells were stained with propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO). Data on viable cells were acquired and analyzed on a FACScan® using Lysys software (Becton Dickinson, San Jose, CA).

Results

Background.   The requirements for stimulating unprimed T cells were examined with the aid of the 2C line of TCR transgenic mice (14). As discussed below, the unique feature of the 2C line is that the TCR specificity of this line and the binding affinities involved in TCR–peptide–MHC interaction are well defined.

2C T cells are MHC class I restricted and undergo positive selection in the thymus to K^b molecules (21). At the level of mature T cells, CD8$^+$ 2C cells display strong alloreactivity to L^d and give high primary proliferative responses and CTL activity to L^d (B10.D2) spleen cells in the

absence of added lymphokines (15, 21). The specificity of 2C cells for L^d is directed to a naturally occurring peptide termed p2Ca (16). This 8-mer peptide is derived from a Krebs cycle enzyme, 2-oxoglutarate dehydrogenase (OGDH) (22); p2Ca has intermediate affinity for L^d molecules and, when complexed to L^d, displays high affinity for cell-bound and soluble 2C TCR molecules (23, 24) (Table 1). A 9-mer variant of p2Ca, termed QL9, has 100-fold higher binding affinity for L^d than p2Ca and 10-fold higher affinity for 2C TCR molecules (25); except for one extra amino acid, QL9 has the same sequence as p2Ca, and, like p2Ca, QL9 forms part of the natural sequence of OGDH.

For mature LN T cells, ~98% of CD8+ 2C cells are clonotype positive (1B2+), and ~98% of 1B2+ cells are CD44lo, indicative of a naive phenotype (Fig. 1 a). Expression of TCR α-chain expression by 2C T cells is presumably minimal because 1B2 expression on 2C T cells is uniformly high and homogenous (Fig. 1 b). This applies to both CD8+ and CD8− (CD4β 1B2+) 2C cells; CD8+ and CD8− subsets of 2C T cells exist as distinct nonoverlapping subsets and can be purified by mAb plus C treatment and panning (Fig. 1 b).

Using purified CD8+ and CD8− 2C cells and synthetic p2Ca and QL9 peptides, we examined the requirements for CD8–MHC and CD28–B7 interaction in the primary response of 2C cells. We established previously that these interactions are crucial for the response of 2C cells to normal B10.D2 spleen as APCs, i.e., where the response is directed to L^d plus endogenous p2Ca peptide (15). The question addressed below is whether the requirement for costimulation decreases when the avidity of T–APC interaction is increased, i.e., when APCs are supplemented with exogenous p2Ca or QL9 peptide. We began by using L^d-transfected RMA-S cells as APCs. Unless stated otherwise, the data discussed below refer to responses measured on day 3 of culture. In all cases, the responses were measured in the absence of exogenous lymphokines; i.e., the responses were helper independent.

CTL and Proliferative Responses Elicited by L^d-transfected RMA-S Cells. TAP-2–deficient RMA-S cells cannot load
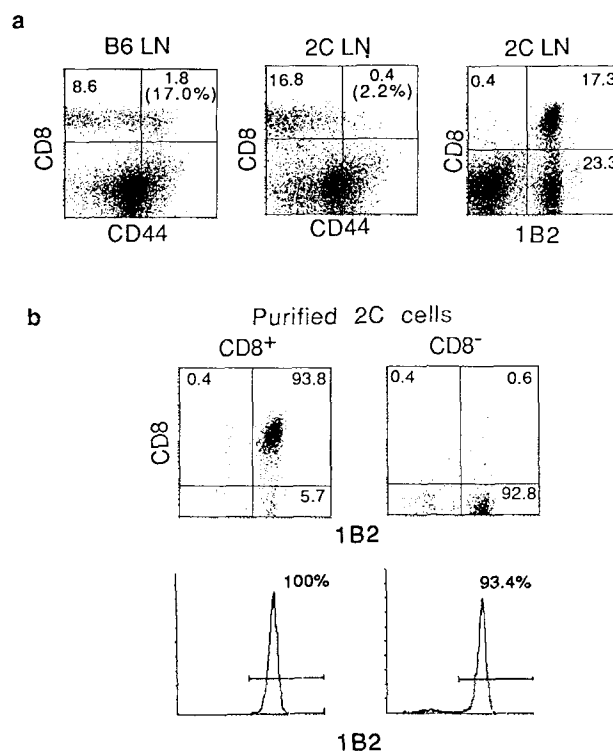
Figure 1. Phenotype of T cells from 2C TCR transgenic mice. (*a*) CD8, CD44, and 1B2 expression on LN cells from 2C TCR transgenic mice compared with their expression in control B6 mice. Freshly isolated LN cells from B6 and 2C TCR transgenic mice were double-stained with mAbs as described in Materials and Methods; the numbers in parentheses refer to the proportion of CD8+ cells that were CD44hi. (*b*) TCR and CD8 expression on purified CD8+ and CD8− 2C T cells. CD8+ and CD8− 2C cells were purified as described in Materials and Methods and double-stained for expression of CD8 and 1B2.

intracellular peptides on to class I molecules: the "empty" class I molecules reach the cell surface, especially at lower temperature, but are unstable and rapidly fall apart (20, 26). However, class I expression on RMA-S cells can be stabilized and increased to a high level by addition of exogenous peptides. Expression of L^d molecules on L^d-transfected RMA-S

Table 1. *Intrinsic Binding Affinities Involved in 2C–Peptide–Ld Interaction, and Stabilization of Ld Expression on RMA-S.Ld Cells by Peptides*

Peptides	Sequences	Affinity for L^d	Affinity of 2C TCR for peptide–L^d	Induction of L^d at 25°C	Stabilization of L^d at 37°C
		M^{-1}	M^{-1}	MFI	MFI
p2Ca	LSPFPFDL	4×10^6	2×10^6	60	7.0
QL9	QLSPFPFDL	4×10^8	2×10^7	63	56.6
MCMV	YPHFMPTNL	2×10^9	NT	38	20.1

The data on peptide sequences and affinity measurements are taken from Sykulev et al. (23, 25). Affinity measurement s for 2C TCR binding to soluble peptide–L^d complexes are based on studies with cell-bound TCR. Experiments of Corr et al. (24) on soluble TCR yielded a somewhat higher affinity measurement for binding to L^d–p2Ca complexes, i.e., 1×10^{-7} M. Induction and stabilization of L^d expression by peptides (10 μM) were measured as described in Materials and Methods; the data refer to the MFI of L^d staining detected with anti-L^d mAb.

cells (RMA-S.L^d) cultured with p2Ca and QL9 peptides is shown in Fig. 2, a and b, and summarized in Table 1. At 25°C, both peptides induced strong L^d expression. However, the concentration of peptides required to induce maximum L^d expression was 4 logs higher for p2Ca than for QL9 (Fig. 2 a). Moreover, only the QL9 peptide caused stable L^d expression at 37°C (Fig. 2 b). In some of the experiments discussed below, a third peptide, MCMV, was used as a control. This peptide binds strongly to L^d (Table 1) but is nonimmunogenic for 2C cells.

RMA-S T lymphoma cells lack B7-1 and B7-2 and show only low levels of ICAM-1 and HSA (Cai, Z., and J. Sprent, unpublished data). RMA-S cells are thus poorly suited to act as APCs for unprimed T cells. To test the APC function of RMA-S cells, CD8$^+$ 2C T cells were cultured with peptide-pulsed irradiated (3,000 cGy) RMA-S.L^d cells in the absence of exogenous lymphokines. The surprising finding was that presentation of p2Ca or QL9 peptide (10 μM) was strongly immunogenic and led to high CTL responses (Fig. 2 c, tested on day 4 with RMA-S.L^d targets) and high proliferative responses (Fig. 2 d, tested on day 3). For proliferative responses (which are easier to quantitate than CTL responses), the QL9 peptide was clearly more immunogenic than the p2Ca peptide, since the dose of peptide required for maximal responses was ∼100-fold higher for p2Ca than for QL9 (Fig. 2 d). The responses to RMA-S.L^d cells were peptide specific, because no response occurred to a third peptide, MCMV (Figs. 2 d and 3 c).

CD8$^-$4$^-$ clonotype-positive (1B2$^+$) cells are a conspicuous population in 2C mice (15, 27) (Fig. 1). In confirmation of previous findings (15), the response of 2C cells to B10.D2 (L^d) spleen cells in the absence of exogenous lymphokines was very high with 1B2$^+$ CD8$^+$ cells but undetectable with 1B2$^+$ CD8$^-$ cells (Fig. 3 a). Very different results were observed with RMA-S.L^d stimulators (Fig. 3, b–d). Here, in the absence of added lymphokines the response to the p2Ca peptide (10 μM) was as high with CD8$^-$ 2C cells as with CD8$^+$ 2C cells. Paradoxically, with the QL9 peptide, responses were substantially higher with CD8$^-$ cells than with CD8$^+$ cells.

At face value, these findings suggest that, in marked contrast with normal B10.D2 spleen cells as APCs, CD8 expression is not required for the response of 2C cells to RMA-S.L^d cells plus exogenous peptides: CD8 expression is redundant for the response to p2Ca and inhibitory for
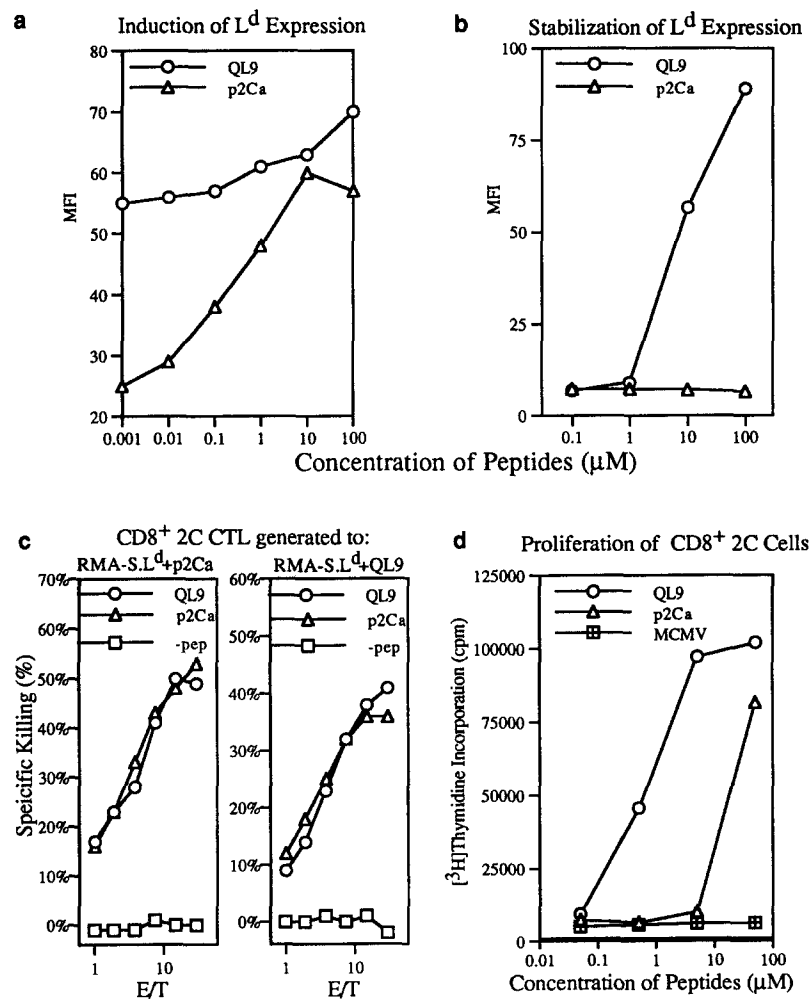

a Induction of Ld Expression

b Stabilization of Ld Expression

c CD8$^+$ 2C CTL generated to:

RMA-S.L^d+p2Ca   RMA-S.L^d+QL9

d Proliferation of CD8$^+$ 2C Cells

Concentration of Peptides (μM)

Figure 2. Functional properties of p2Ca and QL9 peptides. (a) Induction of L^d expression on RMA-S.L^d cells cultured at 25°C overnight in the presence of different concentrations of p2Ca or QL9 peptides; the cells were stained with anti-L^d mAb and analyzed by FACS; the MFI of staining is shown. (b) Stabilization of L^d expression as measured by inducing L^d expression with peptides overnight at 25°C, then raising the temperature to 37°C for 6 h, followed by staining for L^d expression. (c) CTL activity of CD8$^+$ 2C cells elicited by RMA-S.L^d stimulators; CD8$^+$ 2C cells were cultured for 4 d with irradiated (3,000 cGy) RMA-S.L^d cells plus p2Ca or QL9 peptides (10 μM) and then tested for CTL activity on ^{51}Cr-labeled RMA-S.L^d target cells sensitized with p2Ca or QL9 peptides or no peptide (Materials and Methods). (d) Proliferative responses of CD8$^+$ 2C cells to peptides presented by RMA-S.L^d cells; purified LN CD8$^+$ 2C cells (5 × 10^4 cells per well) were cultured with RMA-S.L^d cells (3,000 cGy, 5 × 10^4 cells per well) plus the indicated peptides for 3 d and pulsed with [^{3}H]thymidine during the last 8 h of culture. The data shown are the mean response of triplicate cultures.
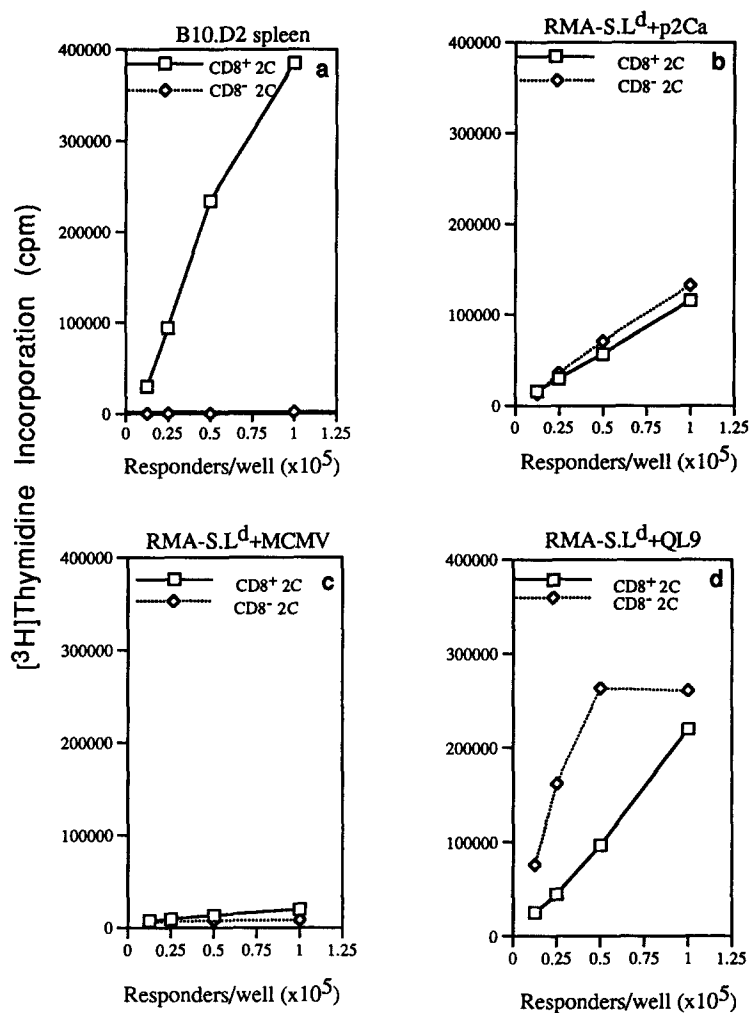
Figure 3. Proliferative responses of CD8+ vs. CD8− 2C cells to RMA-S.L^d cells plus peptides. Purified populations of clonotype-positive (1B2+) CD8+ and CD8− (CD4−) 2C cells prepared from LNs were cultured at 5 × 10^4 cells per well with B10.D2 spleen cells (3,000 cGy; 5 × 10^5 cells per well) without added peptides (*a*), or with RMA-S.L^d cells (3,000 cGy, 5 × 10^4 cells per well) supplemented with the peptides (10 µM) indicated (*b–d*). Cultures were harvested on day 3.

the response to QL9. Further information on the inhibitory effects of CD8 is shown in Fig. 4. With QL9 peptide, it can be seen that CD8− 2C cells gave higher responses than CD8+ 2C cells over a wide range of peptide concentrations, i.e., from 100 µM down to 100 pM (Fig. 4, *a* and *b*). Significantly, adding anti-CD8 mAb to culture markedly enhanced the response of CD8+ cells to QL9 (Fig. 4 *c*). Thus, in the presence of anti-CD8 mAb, responses to QL9 were as high with CD8+ cells as with CD8− cells. Interestingly, the capacity of anti-CD8 mAb to augment the response of CD8+ cells to QL9 also applied to CTLA4Ig (Fig. 4 *d*). This finding was unexpected because RMA-S.L^d cells are B7−. However, staining 2C cells with CTLA4Ig revealed rapid induction of B7 expression after antigen stimulation (data not shown), implying that the enhancing effects of CTLA4Ig reflected binding to B7 on the responder cells. As controls for these experiments, adding anti-L^d mAb to culture inhibited the response of CD8+ 2C cells by 50% (Fig. 4 *d*). Irrelevant mAbs, e.g., anti-CD4, had no effect (data not shown).

The above experiments indicate that helper-independent responses of 2C cells to RMA-S.L^d cells plus QL9 require

neither CD8–MHC interaction nor CD28–B7 interaction. In fact, the costimulation resulting from these interactions is inhibitory. To examine whether this phenomenon applies to conventional APCs, we studied the effects of adding p2Ca and QL9 peptides to normal B10.D2 spleen APCs.

Proliferative Responses to Spleen Cells and DCs. In the absence of exogenous peptide, the response of CD8+ 2C cells to normal B10.D2 spleen cells on day 3 was reduced by ∼80% by CTLA4Ig and by >99% by anti-CD8 or 1B2 mAbs (Table 2). These data with spleen stimulators are consistent with the above findings on CD8+ vs. CD8− 2C cells (Fig. 3 *a*) and reinforce the view that the 2C response to normal B10.D2 spleen cells, i.e., to L^d plus small amounts of endogenous p2Ca, is heavily dependent upon costimulation. When the dose of peptide presented by B10.D2 spleen cells was increased, i.e., by adding exogenous p2Ca peptide, the peak response of CD8+ 2C cells on day 3 was elevated by approximately twofold with 0.1–1.0 µM p2Ca (data not shown). With higher doses of p2Ca, the response declined toward the level found without added peptide. Significantly, as the dose of peptide was raised, the response
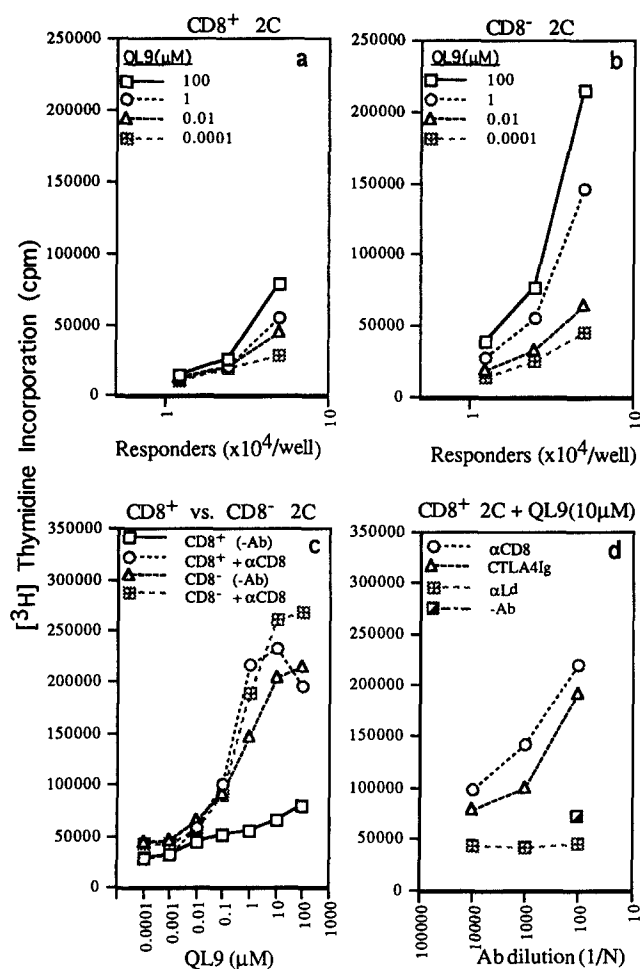
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Figure 4. Blocking costimulation enhances the day 3 proliferative response of CD8+ 2C cells to QL9 peptide presented by RMA-S.L^d cells. (*a* and *b*) Proliferative responses of varying numbers of CD8+ vs. CD8− 2C cells to graded doses of QL9 peptide added to RMA-S.L^d cells. (*c*) Proliferative responses of CD8+ vs CD8− 2C cells (5 × 10^4 cells per well) cultured with QL9-pulsed RMA-S.L^d cells with or without anti-CD8 (αCD8) mAb. (*d*) Proliferative responses of CD8+ 2C cells (5 × 10^4 cells per well) cultured with QL9 (10 μM)–pulsed RMA-S.L^d cells with or without anti-CD8, CTLA4Ig, or anti-L^d mAb. Cultures were harvested on day 3.

became increasingly resistant to inhibition with CTLA4Ig or anti-CD8 mAb. In fact, with high doses of peptide, these reagents augmented the response.

Similar findings were found with the QL9 peptide, but with two differences. First, the dose of QL9 required to augment the response was ~10,000-fold lower than for p2Ca. Second, in contrast with p2Ca, higher doses of QL9 (≥100 nM) reduced the response to below the level found with normal B10.D2 spleen (Table 2). However, adding CTLA4Ig, anti-CD8, or anti-clonotypic 1B2 mAb prevented this reduction and led to very high responses. These findings with B10.D2 spleen plus QL9 are thus in close accord with the above data on RMA-S.L^d cells.

The capacity of high concentrations of QL9 peptide to reduce the response of CD8+ 2C cells to B10.D2 spleen

Table 2. *Proliferative Response of CD8+ 2C Cells to B10.D2 Spleen in the Presence or Absence of QL9 Peptide: Effects of Blocking Costimulation*

| | [³H]Thymidine incorporation (cpm) by CD8+ 2C cells cultured with | |
Addition to Culture	B10.D2 spleen	B10.D2 spleen plus QL9 peptide (10 μM)
Medium alone	198,725	116,348
CTLA4Ig	39,250	234,707
Anti-CD8 mAb	206	195,858
1B2 mAb	140	297,219

Purified CD8+ 2C cells (2.5 × 10^4 cells per well) were cultured with B10.D2 spleen cells (5 × 10^5 cells per well) with or without QL9 peptide (10 μM); CTLA4Ig (1 μg/ml of purified fusion protein), anti-CD8 mAb (1/1,000, ascites fluid), or 1B2 mAb (1;1,000, ascites fluid) was added at the beginning of culture. Cultures were harvested on day 3.

stimulators only applied to high doses of spleen cells. Thus, with lower doses of spleen cells, adding QL9 peptide had the opposite effect and led to enhanced responses (data not shown). These APC dose–dependent effects of QL9 were accentuated when purified B10.D2 DCs were used as a source of APCs. Thus, adding QL9 (10 μM) markedly increased the 2C proliferative response to low doses of B10.D2 DCs but profoundly reduced the response to high doses of DCs (Fig. 5 *a*).

Kinetics of the Response. The above data indicate that the capacity of high doses of QL9 peptide to inhibit the response of CD8+ 2C cells applies to three types of APC, i.e., RMA-S.L^d cells, B10.D2 spleen cells, and B10.D2 DCs. These findings refer to responses measured on day 3 of culture (except for DCs, where responses were measured on day 4). The day 3 time point was chosen because, in the absence of exogenous lymphokines, proliferative responses of normal (nontransgenic) CD8+ T cells to class I alloantigens presented by normal spleen APCs generally reach a peak on day 3 and then fall to low levels on day 4 (28).

As illustrated in Fig. 6 *a*, these kinetics also applied to the proliferative response of CD8+ 2C cells to B10.D2 spleen cells (without added peptides). However, when B10.D2 spleen cells were supplemented with peptides, the kinetics of the 2C response changed considerably. The data discussed below refer to proliferative responses; IL-2 production is discussed later.

With p2Ca peptide, adding high (10 μM) but not low (0.1 μM) concentrations of this peptide to B10.D2 spleen cells prevented the sharp decline in the response on day 4 (Fig. 6, *a* and *b*); this effect was transient, and the response fell to low levels on day 5 (Fig. 6 *b*). The data with QL9 peptide were different (Fig. 6, *a* and *b*). Thus, the inhibition of the response induced by 0.1–10.0 μM QL9 on day 3 changed to a marked increase in the response on day 4; the response then declined on day 5, as with p2Ca peptide.
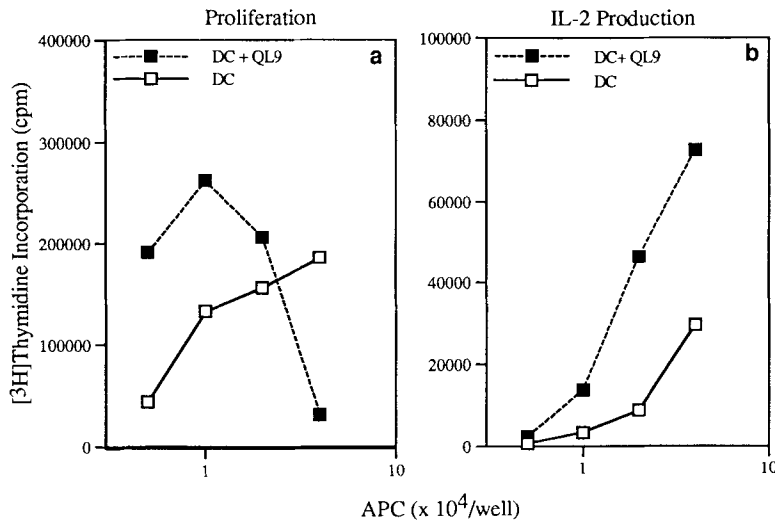
Figure 5. Proliferative responses and IL-2 production by CD8$^+$ 2C cells responding to B10.D2 DCs supplemented with QL9 peptide. CD8$^+$ 2C cells (2.5 × 10^4 cells per well) were cultured with graded doses of DCs in the presence or absence of QL9 peptide (10 μM). Proliferative responses were measured on day 4 (a), and IL-2 production was measured on day 2 (48 h) (b); IL-2 was measured by adding culture supernatant to CTLL-2 indicator cells and, 24 h later, pulsing the indicator cells with [^{3}H]thymidine for 16 h (Materials and Methods).

(Note that in Fig. 6 b, the response to the lower dose of p2Ca peptide was essentially the same as to spleen cells without added peptide; for simplicity, these control data are not shown.)

The capacity of QL9 peptide to augment the late response of 2C cells was more pronounced with DCs than with APCs. Without peptide, the 2C response to B10.D2 DCs reached a peak on day 3 (as with spleen APCs) and then declined to baseline levels by day 5 (Fig. 7). When supplemented with a high dose of QL9 peptide (10 μM), however, the response to DCs was markedly reduced on day 3 and then increased to very high levels on day 5 before declining on day 6. Titration experiments (not shown)

indicated that generation of high responses on day 5 required both a high dose of peptide and a high dose of APCs.

The above data indicate that the inhibition of proliferation induced by a high dose of QL9 peptide is transient and is followed by heightened responses later in culture. IL-2 production in the cultures is discussed below.

IL-2 Production. In accordance with previous findings (15), in the absence of added peptide, IL-2 production elicited by normal B10.D2 spleen cells or DCs as APCs was low but detectable early in culture (days 1 and 2) and fell to background levels by day 3 (Fig. 8). As shown in Figs. 5–8, the capacity of QL9 peptide to inhibit the early proliferative response of CD8$^+$ 2C cells was not associated with a re-
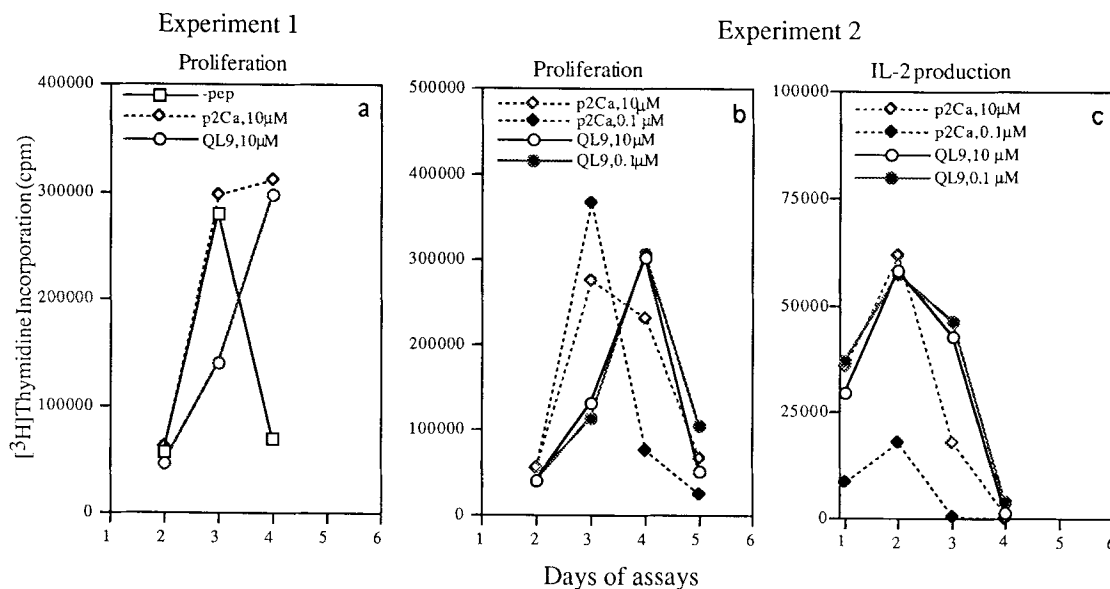

Figure 6. Kinetics of proliferative responses and IL-2 production by CD8$^+$ 2C cells responding to peptides presented by B10.D2 spleen cells. Two experiments are shown; in each experiment, doses of 2.5 × 10^4 CD8$^+$ 2C cells were cultured with 5 × 10^5 B10.D2 spleen cells supplemented with p2Ca or QL9 peptides at the concentrations indicated. In Experiment 1 (a), cultures were harvested on days 2–4 to measure proliferative responses. In Experiment 2 (b and c), cultures were harvested on days 2–5 to measure proliferation and on days 1–4 to measure IL-2 production.
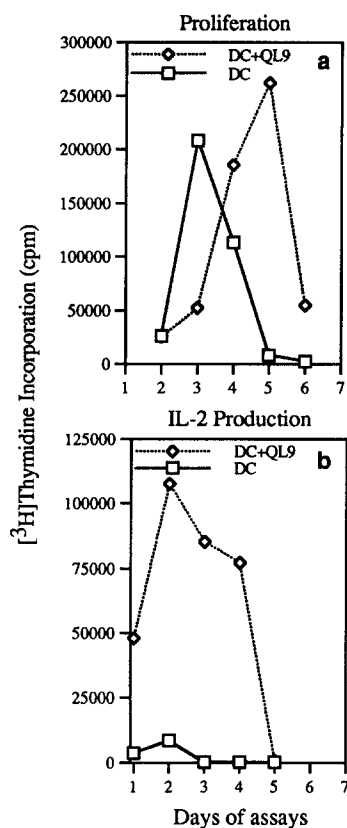
Figure 7. Kinetics of proliferation and IL-2 production by CD8$^+$ 2C cells responding to QL9 peptide presented by B10.D2 DCs. Doses of 2.5 × 10^4 CD8$^+$ 2C cells were cultured with 7 × 10^4 B10.D2 DCs in the presence or absence of QL9 peptide (10 μM). Proliferative responses (a) were measured on days 2–6, whereas IL-2 production (b) was measured on days 1–5.

duction in IL-2 production. In fact, the inhibition of proliferation induced by QL9 was invariably associated with a substantial increase in IL-2 production, both with DCs (Figs. 5 and 7) and spleen cells (Figs. 6 and 8) as APCs. Titration experiments showed that elevation of IL-2 production was both directly proportional to the dose of peptide and dose of APC used, and also considerably stronger with QL9 than p2Ca, especially late in culture (data not shown). In the case of QL9 peptide, IL-2 production remained high until day 3–4 of culture and then declined toward background levels on day 4–5 (Figs. 6 and 7). The late proliferative response elicited by QL9 thus correlated with strong and sustained production of IL-2. In general, the decline in the proliferative response to QL9 was preceded by a fall in IL-2 production 1 d earlier (Figs. 6 and 7).

The influence of costimulation on proliferation vs. IL-2 production is illustrated in Fig. 8, with B10.D2 spleen cells as APCs. As discussed earlier, adding a high dose of QL9 peptide (10 μM) inhibited the proliferative response on day 3 but enhanced the response on day 4; the enhanced proliferative response on day 4 correlated with enhanced IL-2 production on day 2 and day 3. Significantly, blocking costimulation with anti-CD8 mAb or CTLA4Ig reduced the high proliferative response induced by QL9 (10 μM) on day 4

and caused a parallel reduction in IL-2 production 1 day earlier, i.e., on day 3. Thus, in contrast with the early (day 3) proliferative response, the late (day 4) proliferative response to QL9 required costimulation: blocking costimulation reduced IL-2 production earlier in culture (day 3) and thereby abbreviated the proliferative response. Similar findings were found with RMA-S.L^d cells as APCs (data not shown).

The main conclusion from these experiments is that costimulation is essential for the late response of CD8$^+$ cells. Costimulation boosts IL-2 production and thus allows the cells to proliferate for a prolonged period.

Discussion

The main goal of this paper was to examine the primary response of naive CD8$^+$ T cells under various conditions and determine whether the avidity of T cell–APC interaction affects the requirement for costimulation. As discussed earlier, the 2C system is ideally suited for addressing this question because the binding affinities involved in TCR–peptide–MHC interaction are well characterized. With this system, we show here that altering the avidity of T cell–APC interaction induces surprisingly complex changes in the kinetics of the primary responses.

Primary responses of CD8$^+$ cells to class I alloantigens are often undetectable unless the cells receive help in the form of exogenous cytokines. These helper-dependent responses of CD8$^+$ cells reflect low-avidity T cell–APC interactions (15, 29) and are epitomized by the response of CD8$^+$ 2C cells to the weak K^{bm11} alloantigen (15). With stronger alloantigens, the requirement for help does not apply, and CTL and proliferative responses occur in the absence of exogenous lymphocytes. These helper-independent responses can be spectacularly high but are generally of short duration and easily inhibited with anti-CD8 mAb or CTLA4Ig (9, 28). These features apply to the response of CD8$^+$ 2C cells to normal B10.D2 APCs, a situation where the cells react to allo- L^d molecules complexed with small amounts of endogenous p2Ca peptide. We show here that exposure to QL9, a variant of p2Ca with very high affinity for both L^d and the 2C TCR, caused radical changes in the response of CD8$^+$ 2C cells. Adding high concentrations of QL9 peptide to RMA-S.L^d cells, B10.D2 spleen cells, or B10.D2 DCs as APCs led paradoxically to a reduced proliferative response on day 3. However, responses on day 4–5 were markedly elevated. This alteration in kinetics induced by QL9 peptide required costimulation. Thus, preventing costimulation improved the low day 3 proliferative response but reduced the high day 4–5 response. The role of costimulation during the early and late phases of the proliferative response is discussed below.

Reduction of the Response on Day 3. Since peptide-induced inhibition of the CD8$^+$ 2C proliferative response on day 3 was unique to the high affinity QL9 peptide and required large doses of APCs, the inhibition could be a consequence of very high avidity T cell–APC interaction leading to excessive T cell triggering. The observation that blocking co-
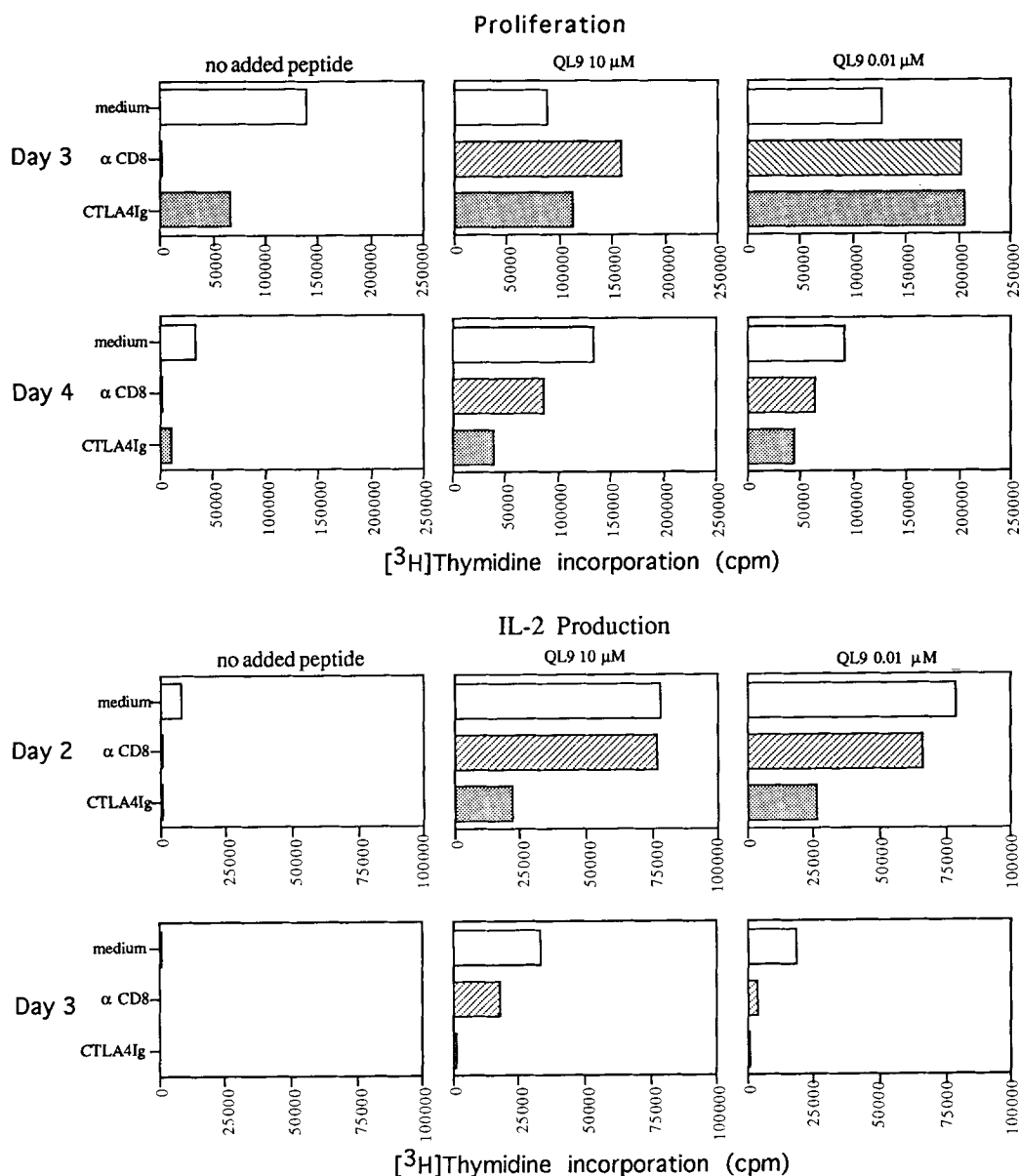
Proliferation

Figure 8. Influence of costimulation on proliferative responses and IL-2 production by CD8+ 2C cells responding to QL9 peptide. Doses of 2.5 × 10⁴ CD8+ 2C cells were cultured with 5 × 10⁵ B10.D2 spleen cells in the presence or absence of QL9 peptide (10 or 0.1 μM) and supplemented with anti-CD8 mAb (1/400 dilution of ascites fluid), CTLA4Ig (4 μg/ml of purified material), or no mAb. Proliferative responses were measured on days 3 and 4, and IL-2 production was measured on days 2 and 3.

stimulation with anti-CD8 mAb or CTLA4Ig overcame the inhibition, presumably by reducing the level of T cell triggering, is consistent with this interpretation. However, explaining the data in terms of excessive T cell triggering is clearly complicated by the sharp increase in the proliferative response after day 3. It is also notable that the inhibition on day 3 applied only to proliferation and not to IL-2 production. Indeed, with high doses of QL9, proliferation and IL-2 production on day 3 seemed to be inversely correlated; thus, blocking costimulation improved the proliferative response but reduced IL-2 production.

The early inhibition of proliferation could be the counterpart of antigen-induced suppression reported for CD4+

cells in vitro (30, 31). In favor of this idea, suppression of CD4+ cells by high doses of antigen is associated with reduced proliferative responses but high IL-2 production. However, the inhibition of proliferation seen in antigen-induced suppression is apparent at both early and late stages of culture and is associated with cell death by apoptosis (31). This clearly contrasts with the present data on CD8+ cells, where the inhibition of proliferation was transient and was followed by very high proliferative responses later in culture. Moreover, examining the cultures on day 3 showed no evidence that the inhibition of proliferation was associated with an increase in cell death (Cai, Z. and J. Sprent, unpublished data). In considering other possibilities, we

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have found that QL9 peptide induces marked TCR down-regulation early in culture (Cai, Z., and J. Sprent, unpublished data). However, the possibility that TCR downregulation accounted for the inhibition of proliferation is unlikely for two reasons. First, TCR downregulation did not impede high IL-2 production. Second, in contrast with the inhibition of proliferation, we have found no evidence that costimulation is required for TCR downregulation.

The precise cause of the inhibition of proliferation induced by QL9 peptide remains unclear. Our working hypothesis is that this phenomenon is a reflection of very strong T cell signaling leading to the transient production of cell cycle inhibitors (32). In this respect, many of the T cells harvested at the stage when the proliferative response is inhibited are locked in G_1 (unpublished data). The level of cell cycle inhibitors in these cells is under investigation.

At face value, the capacity of CTLA4Ig to prevent the inhibition of day 3 proliferative responses is consistent with the evidence that B7 interaction with T cell CTLA4 leads to negative signaling (33, 34). The problem with this interpretation is that the augmentation of the day 3 response by CTLA4Ig also applied to anti-CD8 mAb. Hence, the inhibition of proliferation is more likely to be a reflection of high avidity T cell–APC interaction than selective negative signaling mediated through CTLA4.

Elevation of the Response on Day 4–5. As discussed earlier, typical helper-independent proliferative responses of normal $CD8^+$ T cells are usually of brief duration unless the cultures are supplemented with exogenous IL-2. The capacity of QL9 peptide to elicit high proliferative responses late in culture was thus unexpected.

The kinetics of the late proliferative response to QL9 correlated closely with levels of IL-2 in the cultures. Thus, IL-2 production was low and brief in the absence of QL9 but high and prolonged in the presence of QL9. In each situation, the decline in IL-2 production to baseline levels was followed 1 d later by an abrupt decline in the proliferative response. The capacity of QL9 peptide to induce prolonged proliferation can thus be attributed simply to sustained production of IL-2, presumably as a reflection of strong T cell stimulation. This scenario predicts that reducing the level of T cell stimulation would impair IL-2 production and abbreviate the proliferative response. The effects of blocking costimulation bear out this prediction.

Concluding Comments. A corollary of the avidity model of T cell–APC interaction (8) is that second signals delivered via costimulatory molecules become less important when the avidity of interaction between T cells and APCs is very high. The findings that $B7^-$ RMA-S cells were surprisingly effective APCs for 2C cells and that CTLA4Ig improved rather than reduced the early proliferative response to QL9 are consistent with this prediction. We caution, however, that RMA-S cells do express low levels of ICAM-1 and HSA. Thus, costimulation through these molecules could substitute for CD28–B7 interaction. Moreover, the possibility that RMA-S cells express yet other costimulatory molecules cannot be discounted. Despite this reservation, the data do indicate that costimulation via CD8–class I and CD28–B7 interactions is much less important for responses to strong antigens (B10.D2 APCs plus QL9) than to weak antigens (B10.D2 APCs alone).

Despite these findings on early proliferative responses, costimulation played a crucial role in prolonging the primary response, presumably through intense production of IL-2. Thus, in the absence of costimulation, the primary response to strong antigens, though initially intense, was of brief duration, reflecting rapid consumption of IL-2. The capacity of costimulation to prolong the intensity of the primary response has not been reported previously and adds a new dimension to the importance of costimulation in the immune response.

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