Resting and Activated T Cells Display Different Requirements for CD8 Molecules

By Zeling Cai and Jonathan Sprent

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Summary

Clonotype-positive ($1B2^+$) T cells from 2C T cell receptor (TCR) transgenic mice were used to define the role of CD8 molecules in the induction phase vs. the effector phase of the primary response to class I alloantigens. Three main findings are reported. First, in the presence of exogenous lymphokines, resting $CD8^+$ 2C cells gave strong proliferative responses to two alloantigens, L^d and K^{bm11}. In the absence of added lymphokines, however, $CD8^+$ 2C cells responded only to L^d and not to K^{bm11}; L^d stimulated both interleukin 2 (IL-2) and IL-2 receptor (R) synthesis, whereas K^{bm11} elicited only IL-2R synthesis. The primary response of $CD8^+$ 2C cells was thus helper-independent (HI) to L^d but helper-dependent (HD) to K^{bm11}, presumably reflecting that L^d is a stronger antigen than K^{bm11}. Second, in contrast to $CD8^+$ cells, $CD8^-$ 2C cells mounted only an HD and not an HI response to the strong L^d antigen; conversely, selecting for a minor subset of $CD8^{hi}$ cells enabled 2C cells to mount an HI response to the weak K^{bm11} antigen. These findings, together with experiments with heterozygous vs. homozygous stimulator cells, suggest that HI and HD responses reflect differences in the overall avidity of T antigen presenting cell (APC) interaction: high-avidity interaction leads to strong intracellular signaling and an HI response, whereas low-avidity interaction causes weak signaling and an HD response; high-avidity T/APC interaction is heavily dependent on CD8 expression. Third, CD8 expression was found to be less important for CTL activity than for primary proliferative responses. Thus, in contrast to HI proliferative responses, CTL responses of 2C cells to L^d were CD8 independent. However, 2C lysis of L^d targets became strongly CD8 dependent in the presence of limiting doses of anti-TCR (1B2) antibody. Collectively, the data suggest that, both for T cell induction and the expression of effector function, CD8 molecules play a decisive role in augmenting TCR-mediated signaling, either by promoting TCR contact with antigen or delivering kinases ($p56^{lck}$) to the TCR/CD3 complex, or both.

$CD8^+$ T cells act as precursors of cytotoxic lymphocytes (CTL) and play a key role in controlling T cell responses to viruses (1). $CD8^+$ cells are often viewed as crippled cells that respond poorly to antigen unless supplemented with exogenous IL-2. Under defined conditions, however, it is clear that some $CD8^+$ cells can synthesize their own IL-2 and mount strong responses to antigen in the absence of added lymphokines (2–6). In other situations, however, $CD8^+$ cells fail to synthesize IL-2 and require exogenous IL-2 to respond. The essential difference between these helper-independent (HI)[1] and helper-dependent (HD) subsets of $CD8^+$ cells is controversial. Some workers argue that HI and HD $CD8^+$ cells represent distinct lineages of cells (7, 8). The alternative view is that HI and HD responses are closely related and reflect the avidity of T/APC interaction (6, 9, 10). The suggestion here is that HI responses are a product of high-avidity T/APC interaction, the strength of this interaction being sufficient to trigger synthesis of both IL-2R and IL-2; conversely, HD responses reflect weaker cell interactions which cause synthesis only of IL-2R and not IL-2. TCR transgenic mice provide a useful tool for distinguishing between these two possibilities (see below).

The avidity of T/APC interaction is presumed to be a reflection of many different factors, including the density of the antigen concerned, the intrinsic affinity of the TCR, and the range of complementary accessory/adhesion molecules expressed on T cells and APCs. In the case of $CD8^+$ T cells, the interaction of CD8 molecules with class I molecules on APCs is presumed to play a crucial role in augmenting TCR contact with peptide/class I complexes, thereby enhancing the avidity of T/APC interaction (11–16). This idea has been advanced to explain the finding that, in contrast to typical primary CTL, some $CD8^+$ T cell clones from long-term primed mice can lyse specific target cells in the presence of high concentrations of anti-CD8 mAb (11–13, 17). These "CD8-independent" clones are viewed as a subset of high-

[1] *Abbreviations used in this paper:* CS, culture supernatant; HD, helper-dependent; HI, helper-independent; r, recombinant.

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affinity cells, the intrinsic TCR affinity of these cells being sufficiently high to overcome the need for CD8 molecules to augment T/APC interaction. In addition to acting as adhesion molecules, CD8 molecules are thought to play a key role in T cell triggering (15, 17–19). The role of CD8 in signal transduction is attributed to the capacity of the intracytoplasmic domain of CD8 molecules to associate with the protein tyrosine kinase, $p56^{lck}$. As for the adhesion function of CD8, however, the capacity of CD8 molecules to associate with $p56^{lck}$ does not seem to be mandatory for T cell responses. This is apparent from the finding that $CD8^+$ cells from a transgenic mouse line showing defective CD8/lck association retain the capacity to mount CTL responses (20).

To clarify the role of CD8 molecules in T cell responses, we have examined the function of $CD8^+$ cells from the 2C line of TCR transgenic mice (21–24); this line undergoes positive selection to H-2 class I K^b molecules in B6 mice and shows strong alloreactivity to L^d molecules and weaker reactivity to K^{bm3} (bm3) and K^{bm11} (bm11) molecules. With this line we show here that unprimed B6 2C cells mount typical HI responses to strong alloantigens (L^d stimulator cells) but HD responses to weak alloantigens (bm11 stimulators); this finding rules out the possibility that HI and HD responses of $CD8^+$ cells involve different lineages (see above). With this background, we present evidence that the role of CD8 in T cell activation is complex and depends on both the activation status of the responding cells and the strength of the allo-stimulus. The key finding is that CD8-independent responses of 2C cells to strong antigens can be converted to CD8-dependent responses by impairing TCR contact with antigen. We interpret this finding to indicate that the primary function of CD8 molecules is to augment weak TCR-mediated signals.

Materials and Methods

Mice. 2C TCR transgenic mice were kindly provided by Dr. D. Loh (University of Washington, St. Louis, MO) (21). C57BL/6 (B6), B10.D2/NSnJ (B10.D2), B6.C-H-2^{bm1}/ByJ (bm1), B6.C-H-2^{bm3}/EgAoEg (bm3), B6.C-H2^{bm11}/Melvold (bm11), and (B6 × bm3)F_1 and (B6 × bm11)F_1 mice were obtained from The Scripps Research Institute breeding facility.

mAbs. The following mAbs were used: 53-6.7 and 3.168.8 (anti-CD8) (25, 26); RL172 (anti-CD4) (27); J11d (anti–heat stable antigen) (28); 28-16-8s (anti-IA^b) (29); FD441.8 (anti-LFA-1) (30); and 7D4 (anti-IL-2Rα) (31). 1B2, the clonotypic mAb for 2C TCR, was kindly provided by Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA) (32). CTLA4Ig fusion protein was a gift of Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland) (33). 1B2 Fab fragments were prepared by papain digestion followed by purification with a protein A column (Pierce Chemical Co., Rockford, IL).

Media. As described previously (3), HBSS supplemented with 2.5% γ-globulin-free horse serum (Gibco Laboratories, Santa Clara, CA) was used for preparation of single cell suspensions. For MLR, 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. For generation of CTL, DMEM supplemented with 1 mM sodium pyruvate and the above supplements was used. Exogenous lymphokines, in the form of recombinant (r)IL-2 (Cetus Corp., Emeryville, CA) or culture supernatant (CS) from stimulated EL4 cells (1,000 U IL-2/ml), were added in some experiments (34).

Purification of $1B2^+$ Cells. Lymphocyte cell suspensions were prepared from pooled cervical, axillary, inguinal, and mesenteric LNs of 2C TCR transgenic mice. Cells were purified by first treating 2C LN cells with a cocktail of mAbs (anti-CD4, anti–heat stable antigen, anti-$I\text{-}A^b$) plus C for 45 min at 37°C; >90% of the surviving cells were $1B2^+$. These cells were further separated into $CD8^+$ and $CD8^-$ ($CD4^-$) cells by panning at 4°C for 90 min on petri dishes coated with anti-CD8 mAb (3). Nonattached cells were eluted and treated with anti-CD8 mAb and C to obtain $CD8^-1B2^+$ cells. The attached ($CD8^+$) cells were recovered by incubation at 37°C for 5 min followed by vigorous pipetting.

MLR. As described previously (3), unless stated otherwise 10^5 responder cells were cultured in flat-bottom microtiter plates with 5×10^5 2,000-cGy–irradiated spleen cells as stimulators in a volume of 200 μl. Cultures were pulsed with 1 μCi [^{3}H]TdR and harvested 8 h later. All of the data shown in the figures refer to the mean of triplicate cultures. SD were generally within 5–15% of the mean.

IL-2 Synthesis. Purified $1B2^+CD8^+$ 2C cells (10^5) were cultured with 5×10^5 irradiated spleen cells in the presence of anti-IL-2Rα mAb (7D4). Supernatants were collected at various time points and tested for IL-2 activity using an IL-2–dependent cell line CTLL (35).

Generation of CTL and CTL Assays. 10^6 $CD8^+1B2^+$ 2C cells or 2×10^6 $CD8^-1B2^+$ cells were cultured with 5×10^6 2,000-cGy–irradiated spleen cells in a volume of 2 ml in a 24-well plate; with $CD8^-$ responder cells, the cultures were supplemented with 2% EL4 CS (34). After 4–5 d, the cells were pooled and adjusted to the required number. To prepare target cells, spleen cells were stimulated with 2.5 μg/ml Con A supplemented with 5% EL4 CS. After 64–68 h, Con A blasts were harvested and labeled with ^{51}Cr (100 μCi/2×10^6 cells) at 37°C for 90 min, and then were washed thoroughly. To measure CTL activity, varying numbers of CTLs were cultured with 10^4 ^{51}Cr-labeled targets in the presence or absence of mAbs for 4 h. Supernatants were harvested to measure ^{51}Cr release. All of the data refer to the mean of duplicate cultures; SD were within 5–15% of the mean. Percent specific lysis was calculated as: 100 × [(experimental − spontaneous ^{51}Cr release)/(total − spontaneous ^{51}Cr release)]. For inhibition experiments by mAbs, the results were expressed as percent inhibition calculated as: [1 − (% lysis with mAb/% lysis without mAbs)] × 100.

FACS® Analysis and Cell Sorting. Purified fresh $CD8^+1B2^+$ or $CD8^-1B2^+$ 2C cells, or $1B2^+$ 2C cells cultured with spleen stimulators and then purified on Histopaque 1083 (Sigma Chemical Co., St. Louis, MO) gradients, were stained with 1B2 mAb followed by FITC-conjugated goat $F(ab)'_2$ anti-mouse Fcγ antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). After blocking of free binding sites with normal mouse serum, cells were stained with PE-conjugated anti-IL-2Rα mAb (7D4) or PE-conjugated anti-CD8 mAb (GIBCO BRL, Gaithersburg, MD). Dead cells were stained with propidium iodide (Sigma Chemical Co.). Viable stained cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). For cell sorting, purified $1B2^+$ 2C cells were stained with PE-conjugated anti-CD8 mAb (GIBCO BRL) and sorted for $CD8^{hi}$ and $CD8^{lo}$ cells on FACSstar® flow cytometer (Becton Dickinson & Co.).

Results

Experimental Approach. Purified $CD8^+$ T cells were prepared from LN of 2C B6 mice by a combination of mAb

plus complement treatment followed by positive panning for $CD8^+$ cells (see Materials and Methods). When stained with 1B2 anticlonotypic mAb, >95% of 2C $CD8^+$ cells were $1B2^+$. Nearly all of these cells were naive resting cells, the proportion of activated (e.g., $Pgp\text{-}1^{hi}$) cells being <5%. In initial experiments, unprimed 2C $CD8^+$ cells were tested for their capacity to mount primary proliferative responses to alloantigens (2,000-cGy spleen cells) with or without added lymphokines. Proliferation was measured by adding [^{3}H]thymidine to the cultures 8 h before harvest.

2C $CD8^+$ Cells Give HI Responses to L^d Stimulators but HD Responses to bm11 Stimulators. In the absence of added lymphokines, small doses of 2C $CD8^+$ cells (10^5) produced very strong proliferative responses to stimulator cells expressing L^d alloantigens (B10.D2 spleen) (Fig. 1 *a*); as measured by [^{3}H]thymidine incorporation, responses reached a peak on day 3 (>200,000 cpm) and then declined precipitously. In contrast to the strong response of 2C cells to L^d, 2C cells gave a much lower response to bm3 and almost no response to bm11 (Fig. 1 *a*). In the presence of exogenous lymphokines enriched in IL-2 (EL4 CS), however, 2C cells responded almost as effectively to bm3 and bm11 as to L^d (Fig. 1 *b* and not shown); background responses to syngeneic B6 stimulators were very low (Fig. 1 *b*). The limited response of 2C $CD8^+$ cells to bm3 and bm11 stimulators in the absence of added lymphokines correlated with poor IL-2 production. Thus, in marked contrast to the high IL-2 production elicited by L^d stimulators, 2C $CD8^+$ cells produced only low levels of IL-2 in response to bm3 and almost no IL-2 to bm11 (Fig. 1 *c*).

Collectively, these data indicate that 2C cells show conspicuous qualitative differences in their response to different alloantigens. For L^d antigens, the response of 2C cells is associated with high IL-2 production, and this allows the responding cells to mount strong proliferative responses in the absence of exogenous lymphokines. The 2C response to L^d is thus a typical HI response (see above). For bm11 antigens, by contrast, 2C cells fail to synthesize IL-2 and cannot mount proliferative responses unless supplemented with exogenous lymphokines. The 2C response to bm11 is thus limited to an HD response, HI responses being almost undetectable. bm3 antigens elicit intermediate responses: 2C cells give a definite HI response to bm3 but this response is much lower than to L^d.

Influence of Antigen Dose. The above data refer to the response of 2C cells to limiting doses of APCs. With high doses of APCs (10^6 homozygous spleen cells), 2C cells gave moderately strong HI responses to bm3 and weak but significant responses to bm11 (Fig. 2 *a*). This finding implies that HI and HD responses are closely related, and that increasing the dose of antigen can convert an HD response to an HI response and vice versa. To explore this idea we compared the response of 2C cells with homozygous vs. heterozygous bm3 and bm11 stimulator cells. As shown in Fig. 2 *b*, HI responses of 2C cells to bm3 APCs were substantial with homozygous stimulators but very low with heterozygous stimulators. Likewise, the minimal response of 2C cells to homozygous bm11 stimulators was reduced to background levels when heterozygous stimulators were used (data not shown). The minor (twofold) difference in antigen density on homozygous vs. heterozygous stimulators thus had a major effect on the intensity of the HI response. This was much less evident for HD responses because, in the presence of EL4 CS, both heterozygous and homozygous stimulators led to strong responses of 2C cells (data not shown).

IL-2R Expression. Although HD responses of 2C cells to L^d, bm3, and bm11 were uniformly high to each antigen, this only applied when exogenous lymphokines were added in large doses (Fig. 1 *b*). With lower doses of lymphokines, responses to bm11 stimulators were substantially lower than to L^d. To investigate this question further, we examined IL-2R expression on 2C cells. 2C cells were cultured with high doses of homozygous L^d, bm3, or bm11 stimulators in the absence of lymphokines, and IL-2R expression was studied after 24 or 48 h (Fig. 3). With L^d (B10.D2) stimulators, IL-2R expression was prominent at 24 h (85% positive cells) and complete by 48 h (98%). With bm11 stimulators, by contrast, IL-2R expression was very limited at 24 h (20%); IL-2R expression was much higher at 48 h (76%) but the density of expression was appreciably lower than with L^d

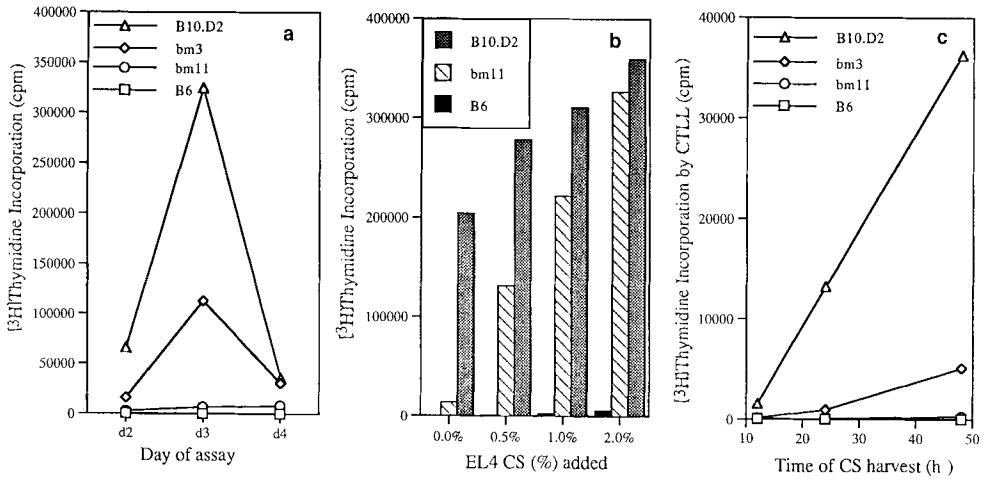

Figure 1. Proliferative responses of purified $CD8^+1B2^+$ 2C cells to class I alloantigens. (*a*) Proliferative responses in the absence of exogenous cytokines; 10^5 2C responder cells were cultured with 5×10^5 irradiated B10.D2, bm3, bm11, or B6 spleen stimulator cells for 2, 3, or 4 d before addition of [^{3}H]TdR. (*b*) Proliferative responses to class I alloantigens in the presence of exogenous cytokines (EL4 CS); cultures were harvested at day 3. (*c*) IL-2 production by 2C cells; 10^5 responder cells were cultured with 5×10^5 spleen stimulator cells and IL-2 levels in the culture supernatant were measured at the times indicated (Materials and Methods).

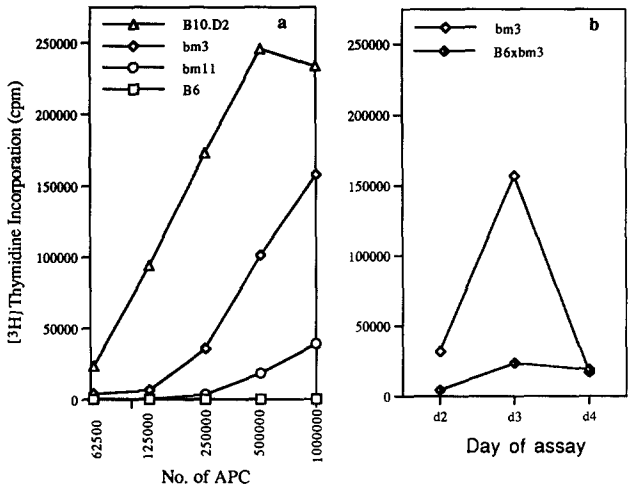

Figure 2. Influence of antigen dose and density on proliferative responses of CD8+1B2+ 2C cells. (*a*) Proliferative responses of 10^5 2C responder cells cultured for 3 d with varying numbers of irradiated B10.D2, bm3, or bm11 spleen stimulator cells. (*b*) Proliferative responses of 10^5 2C cells cultured with 5×10^5 irradiated homozygous bm3 or heterozygous (B6 × bm3) F_1 spleen stimulator cells for 2, 3, or 4 d.

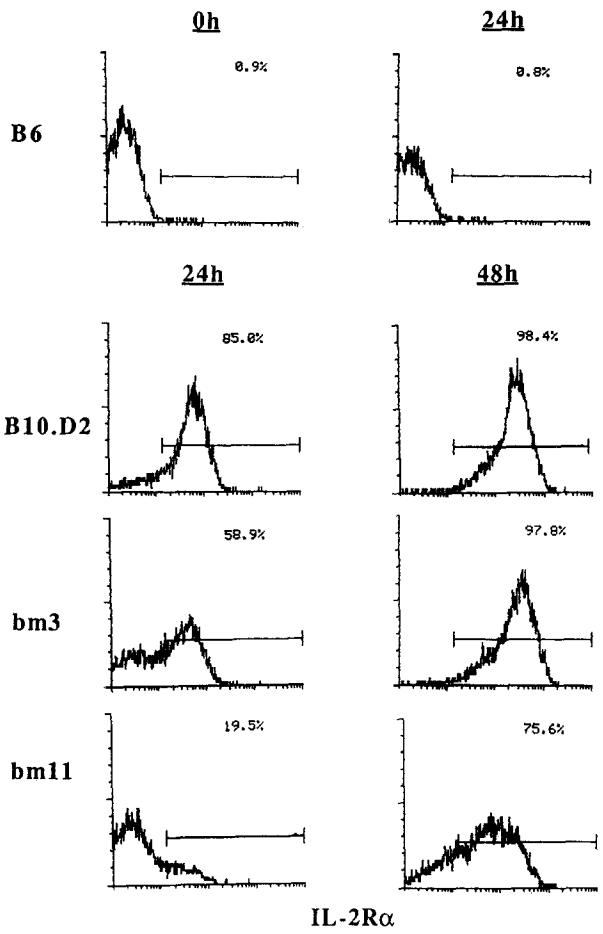

Figure 3. Expression of IL-2Rα on CD8+1B2+ 2C cells cultured with B10.D2, bm3, bm11, or B6 spleen cells. Purified 2C cells were stimulated with irradiated spleen stimulator cells for 24 h or 48 h, and double stained with 1B2 and anti-IL-2Rα mAbs. The data show IL-2Rα expression on 1B2+ cells; dead cells were gated out by propidium iodide staining.

stimulators. With bm3 stimulators, IL-2R expression was intermediate (59% at 24 h, 98% at 48 h). This hierarchy in IL-2R expression elicited by L^d, bm3 and bm11 stimulators thus correlates closely with the intensity of the 2C HI proliferative response to these three antigens. In addition, the retarded and incomplete expression of IL-2R induced by bm11 stimulators explains why HD responses to bm11 are suboptimal unless exogenous lymphokines are added in high doses (see above).

Inhibition of 2C Cells with Anti-CD8 Antibody. The hierarchy in the response of 2C cells to L^d, bm3 and bm11 stimulators also correlated with susceptibility to inhibition with anti-CD8 mAb. In the absence of added lymphokines, anti-CD8 mAb completely inhibited the response of 2C cells to all three antigens. Different results were obtained for HD responses, i.e., when the cultures were supplemented with lymphokines (rIL-2) (Fig. 4 *a*). In this situation, anti-CD8 mAb caused substantial (80%) inhibition of the response to the L^d antigen (measured on day 3), but only when high doses of anti-CD8 mAb were used. By contrast, even low doses of anti-CD8 mAb caused complete inhibition of the response to bm3 and bm11 antigens, the response to bm3 being slightly less sensitive to inhibition than the response to bm11. This hierarchy of susceptibility to inhibition with anti-CD8 mAb did not apply to CTLA4Ig (which blocks T cell contact with B7 molecules on APCs [33]) (Fig. 4 *b*) or to anti-LFA-1 mAb (data not shown). With these reagents, all three responses were inhibited equally.

The CD8⁻ Subset of 2C Cells Gives Only an HD Response to L^d Stimulators. To rule out the possibility that the inhibitory effects of anti-CD8 mAb reflected negative signaling, we examined the response of the CD8⁻ (CD4⁻) subset of 1B2+ 2C cells. CD8⁻ IB2+ cells represent a discrete subset (see below, Figs. 7 *a* and 8, *bottom right*); CD8 expression on CD8⁻ IB2+ cells is no higher than on B cells and this phenotype is stable in culture. The experiment shown in Fig. 5 compares the response of CD8+ vs. CD8⁻ 1B2+ 2C cells with L^d antigen in the presence or absence of IL-2 (EL4 CS); responses were measured on days 2, 3, and 4. For CD8+ 2C cells, the high HI response to L^d (maximal on day 3) was completely eliminated by anti-CD8 mAb and partly restored when anti-CD8 mAb was supplemented with IL-2; responses with IL-2 were low on day 3 (indicative of partial inhibition of the HD response; Fig. 4) but reached high levels on day 4. Unlike CD8+ cells, CD8⁻ 2C cells gave no detectable response to L^d unless supplemented with IL-2. In the presence of IL-2, however, CD8⁻ 2C cells responded quite well to L^d, the response on day 3 being comparable to the day-3 response of CD8+ 2C cells supplemented with IL-2 plus anti-CD8 mAb (Fig. 5). These findings indicate that the inhibitory effects of anti-CD8 mAb are not a reflection of negative signaling. In addition, the data confirm that CD8 expression is obligatory for HI responses: in the absence of CD8 (or when CD8 is blocked), the response of 2C cells to L^d is converted to an HD response.

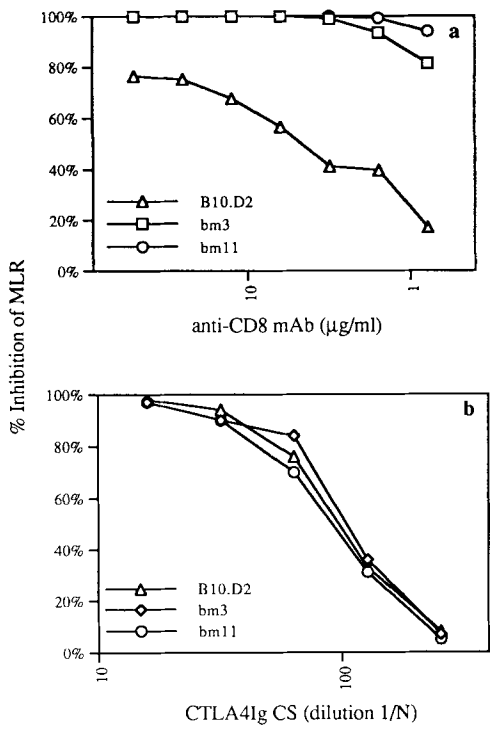

Figure 4. Inhibition of HD proliferative responses of CD8$^+$1B2$^+$ 2C cells to L^d, K^{bm3}, and K^{bm11} antigens by anti-CD8 mAb and CTLA4Ig. 10^5 2C cells supplemented with IL-2 (15 U rIL-2) were cultured with 5 × 10^5 irradiated spleen stimulator cells for 3 d. The cultures were supplemented with different concentrations of anti-CD8 mAb (53-6.7) (*a*) or CTLA4Ig (*b*). The results are expressed as percent inhibition calculated as: 100 × [1−([^{3}H]TdR incorporation with mAb/[^{3}H]TdR incorporation without mAb)].

The finding that the CD8$^-$ subset of 2C cells mounted only an HD and not an HI response to L^d raised the question whether CD8$^-$ 2C cells would retain reactivity to bm11. Interestingly, in contrast to CD8$^+$ 2C cells (Fig. 1 *b*), CD8$^-$ 2C cells displayed total unresponsiveness to bm11 (Fig. 6). Thus, even in the presence of exogenous IL-2 the response of CD8$^-$ 2C cells to bm11 (and also to bm3) was no higher than the background response to syngeneic B6 stimulators.

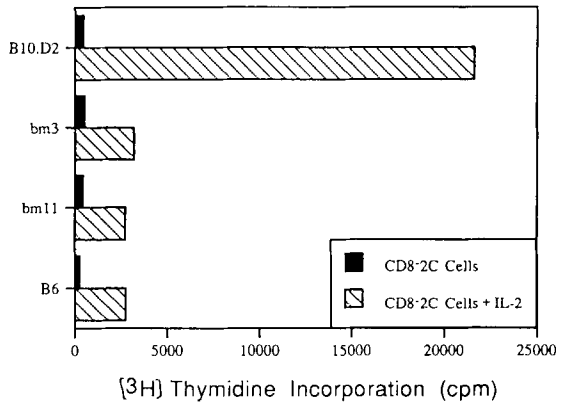

Figure 6. Failure of CD8$^-$ 2C cells to give HD proliferative response to K^{bm3} and K^{bm11} antigens. 10^5 purified CD8$^-$1B2$^+$ 2C cells were cultured with 5 × 10^5 irradiated B10.D2, bm3, bm11, or B6 spleen cells for 4 d in the presence or absence of IL-2 (40 U rIL-2).

High CD8 Expression Accentuates the HI Response. Further information on the role of CD8 molecules in controlling primary responses of 2C cells was obtained by separating by FACS® the CD8$^+$ population of 2C cells into two subsets of cells expressing the highest vs. the lowest density of CD8 (Fig. 7 *a*). Each of these subsets represented 5% of total CD8$^+$ cells. The level of CD8 expression on these two populations is shown in Fig. 7 *b*; the density of CD8 on the CD8^{hi} cells was about threefold higher than on the CD8^{lo} cells.

Unlike CD8$^-$ 2C cells (Fig. 5), the CD8^{lo} subset of 2C cells gave moderately strong HI responses to L^d stimulators (Fig. 7 *c*); responses were higher with the CD8^{hi} subset, but only by a factor of twofold. Very different results occurred with bm3 and bm11 stimulators (Fig. 7, *d* and *e*). With these stimulators, CD8^{lo} 2C cells gave barely detectable HI responses. The CD8^{hi} cells, however, gave surprisingly strong responses, even to bm11. These findings reenforce the view that HI and HD responses are closely related and indicate that a comparatively minor (threefold) increase in CD8 density is sufficient to convert an HD response to a strong HI response.

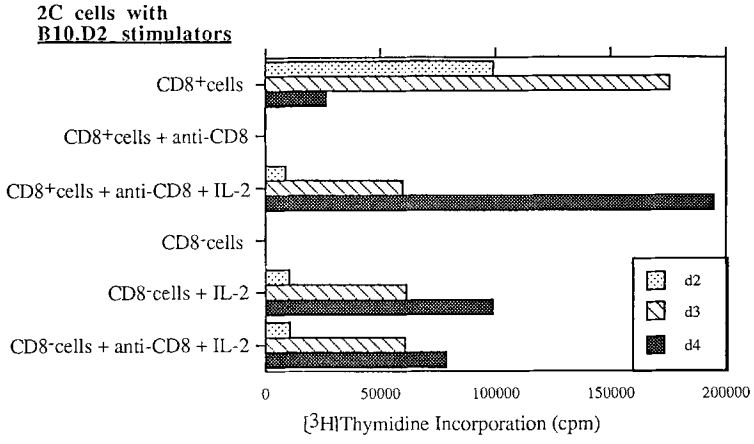

Figure 5. Requirement for CD8 molecules in HI vs. HD proliferative responses of 1B2$^+$ 2C cells to L^d antigen. 10^5 purified CD8$^+$ or CD8$^-$ 2C cells were cultured with 5 × 10^5 irradiated B10.D2 spleen cells for 2, 3, or 4 d. Anti-CD8 mAb (3.168) and IL-2 (2% EL4 CS) were added as indicated.

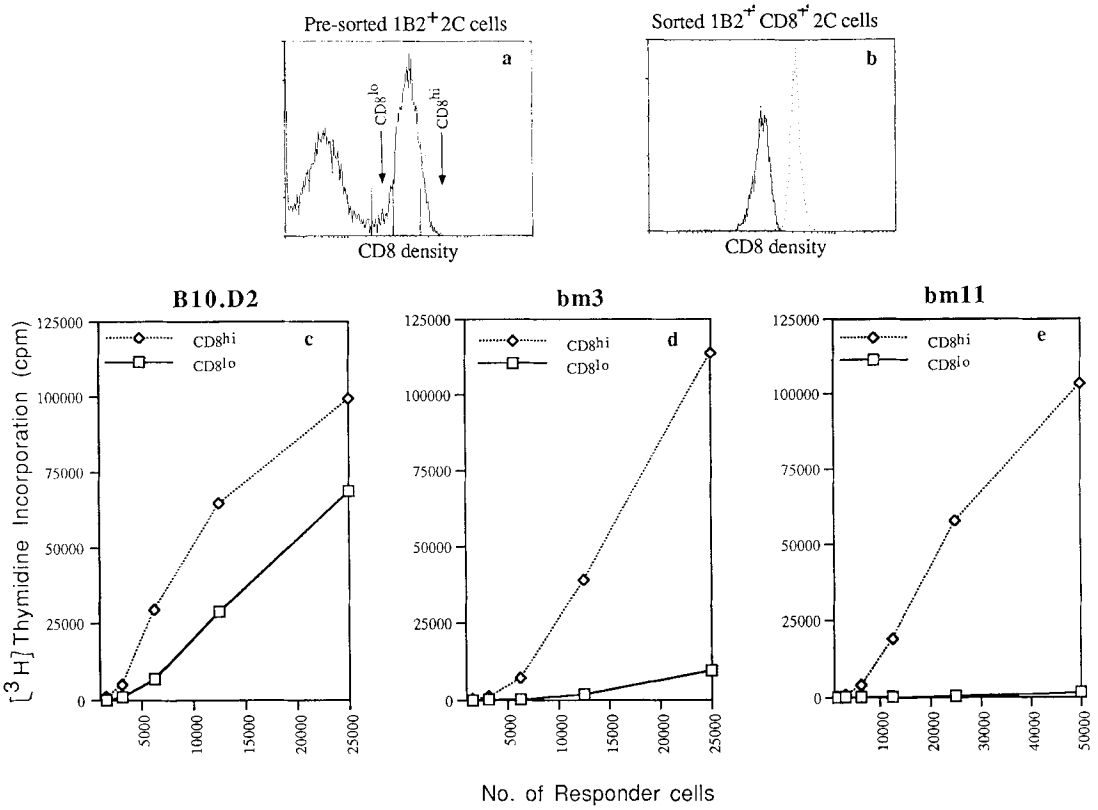

Figure 7. Influence of CD8 density on HI proliferative responses of 2C cells. (*a*) CD8 expression on unseparated 2C cells. (*b*) CD8 expression on CD8+ 2C cells sorted into two populations of $CD8^{hi}$ and $CD8^{lo}$ cells. (*c–e*) HI proliferative responses of $CD8^{hi}$ vs. $CD8^{lo}$ 2C cells cultured with 5×10^5 irradiated B10.D2 (*c*), bm3 (*d*), or bm11 (*e*) spleen cells for 3 d.

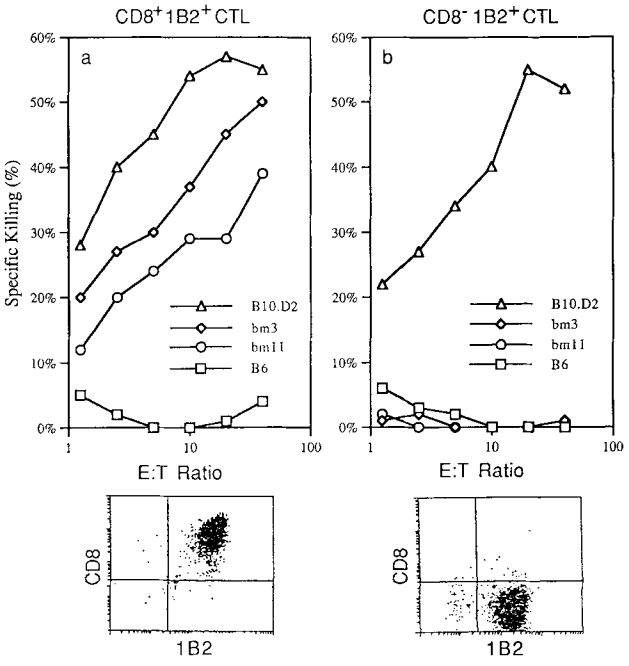

Figure 8. Cytolytic activity of CD8+ vs. CD8− 1B2+ 2C CTL. Purified CD8+ and CD8− 2C cells were cultured with irradiated B10.D2 spleen cells for 5 d to generate CTL; with CD8− responders, the cultures were supplemented with 2% EL4 CS. The two populations of stimulated CD8+ (*a*) and CD8− (*b*) 2C cells were then tested for their capacity to lyse B10.D2, bm3, bm11, and B6 target cells (^{51}Cr-labeled Con A blasts) in a 4-h ^{51}Cr release assay (Materials and Methods). The two panels at the bottom of the figure show the FACS® profile of the two populations of CTL used.

Role of CD8 Molecules during CTL Activity. All of the above data refer to primary proliferative responses of resting 2C cells. To examine the role of CD8 molecules in the effector phase, 2C cells were separated into subsets of CD8+ 1B2+ cells and CD8− 1B2+ cells (Fig. 8, *bottom*). These cell populations were then cultured with L^d (B10.D2 spleen) for 4 d to generate CTL; for the CD8− cells, it was necessary to supplement the cultures with lymphokines (EL4 CS). The CTL activity of the two populations of cells is shown in Fig. 8 (*top*).

The CD8+ cells showed a hierarchy of CTL activity, with strong lysis of L^d targets, slightly lower lysis of bm3 targets and intermediate lysis of bm11 targets. In marked contrast to CD8+ CTL, the CD8− subset of 2C cells failed to lyse either bm3 or bm11 targets. Lysis of L^d targets, by contrast, was almost as high as with CD8+ CTL. These findings indicate that CD8 expression is not required for lysis of L^d targets but is essential for lysis of bm3 and bm11 targets.

To seek further information on this issue, we examined the susceptibility of CD8+ 2C CTL to inhibition with anti-

CD8 mAb. As shown in Fig. 9 *a*, adding anti-CD8 mAb to CD8$^+$ 2C CTL failed to inhibit lysis of L^d targets. By contrast, anti-CD8 mAb substantially reduced lysis of bm3 targets and markedly inhibited lysis of bm11 targets. These findings are thus in close agreement with the above data on CD8$^-$ CTL. As controls for these experiments we used anti-LFA-1 mAb and CTLA4Ig CS. In contrast to anti-CD8 mAb, anti-LFA-1 mAb inhibited lysis of all three targets equally (Fig. 9 *b*). CTLA4Ig CS caused no inhibition of lysis (Fig. 9 *c*).

2C Lysis of L^d Targets Becomes CD8 Dependent when TCR Contact with Antigen Is Inhibited. The simplest explanation for the finding that 2C lysis of L^d targets is CD8 independent is that L^d is a "strong" antigen, the affinity of the 2C TCR for L^d being sufficiently high to bypass the requirement for CD8 expression (see Discussion). Conversely, the CD8 dependency of 2C lysis to bm3 and bm11 antigens implies that these are weak antigens which cannot be recognized functionally by the 2C TCR without coinvolvement of the CD8 molecule. This line of reasoning raises the question whether decreasing TCR contact with antigen, e.g., with anti-TCR antibody, would convert a strong (CD8-independent) antigen such as L^d to a weak (CD8-dependent) antigen.

We examined this possibility with the aid of 1B2 (anticlonotype) mAb, using Fab fragments. Initial experiments established that, at high concentrations, 1B2 Fab fragments were able to cause strong inhibition of lysis of L^d targets by CD8$^+$ 2C CTL (Fig. 10). Based on this finding, a limiting dose of 1B2 Fab was then added to 2C CD8$^+$ CTL ± anti-CD8 mAb (Fig. 11 *a*). In the absence of anti-CD8 mAb a small dose of 1B2 caused minimal inhibition (< 10%). Supplementing limiting doses of 1B2 with increasing concentrations of anti-CD8 mAb, however, caused strong inhibition of lysis.

These observations verify the prediction that CTL activity to a strong antigen becomes CD8 independent when TCR contact with antigen is impaired. The question then arises whether the reverse situation would apply i.e., that inhibiting CD8 function would augment sensitivity to inhibition with 1B2. To assess this possibility, CD8$^+$ 2C CTL were cultured with L^d targets in the presence of increasing doses of 1B2 ± a high dose of anti-CD8 mAb. As shown in Fig. 11 *b*, addition of anti-CD8 mAb markedly increased the sensitivity of 2C CTL to inhibition by 1B2. Thus, 2C CTL supplemented with anti-CD8 mAb were 40-fold more sensitive to inhibition with 1B2 than 2C cells cultured with 1B2 alone.

In light of this finding, the prediction followed that the

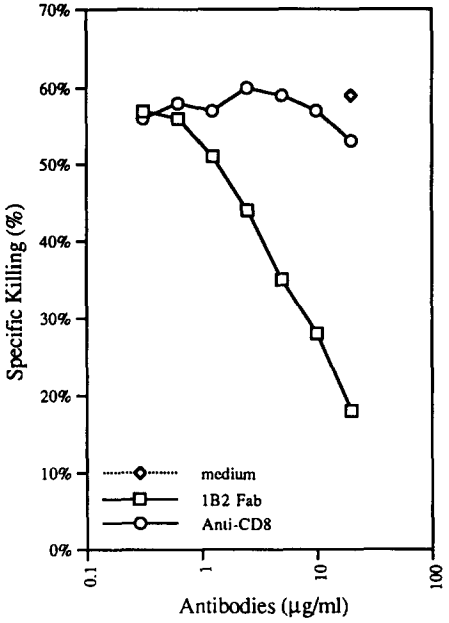

Figure 10. Capacity of 1B2 Fab fragments vs. anti-CD8 mAb to inhibit lysis of B10.D2 target cells by CD8$^+$ 2C cells. As for Fig. 9, activated CD8$^+$ 2C CTL were cultured with ^{51}Cr-labeled B10.D2 target cells at a 20:1 ratio in medium alone or together with increasing concentrations of 1B2 Fab fragments or anti-CD8 mAb (53-6.7).

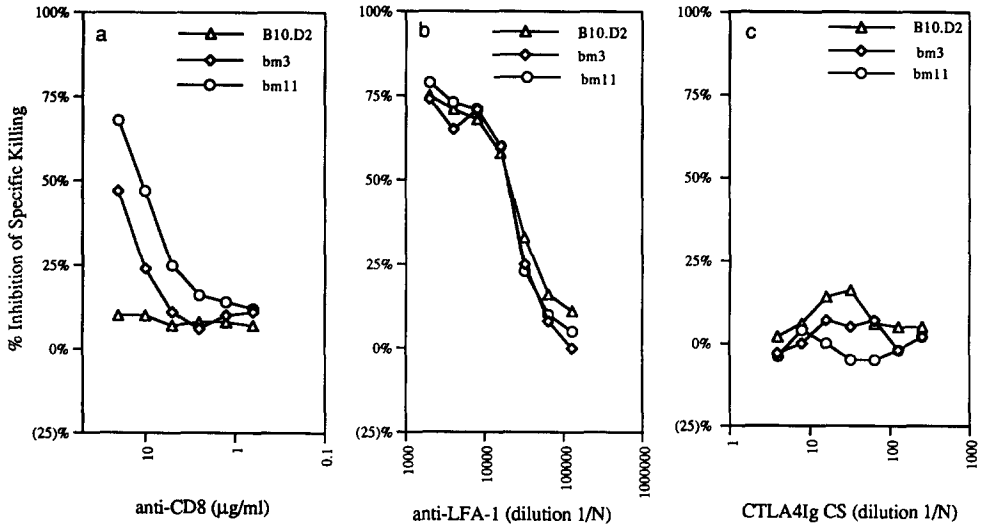

Figure 9. Inhibition of CTL activity of CD8$^+$1B2$^+$ 2C cells by anti-CD8 mAb (53-6.7) (*a*), anti-LFA-1 mAb (*b*), and CTLA4Ig (*c*). Using a 20:1 ratio, 2 × 10^5 CD8$^+$1B2$^+$ CTL were cultured with 10^4 ^{51}Cr-labeled B10.D2, bm3, or bm11 target cells for 4 h in the presence or absence of the indicated mAbs. Percent inhibition of specific killing was calculated as described in the Materials and Methods.

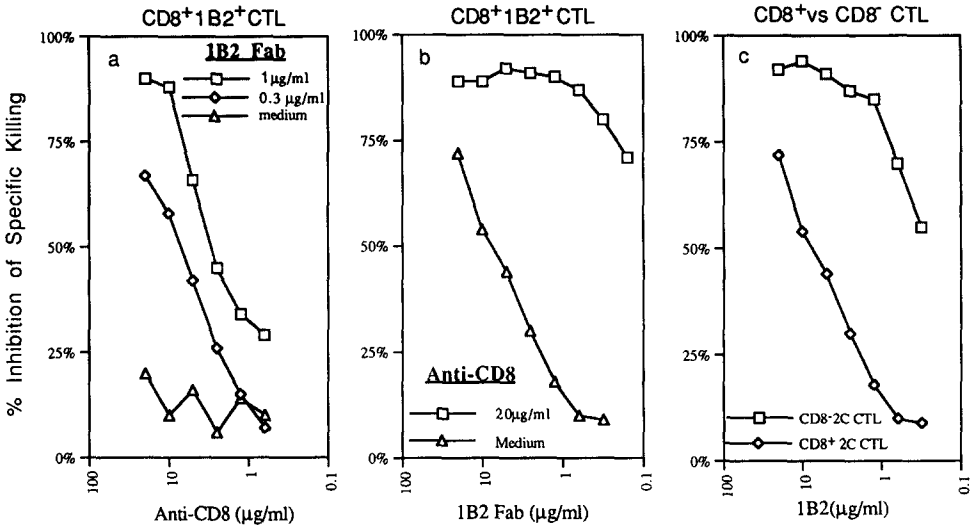

Figure 11. Synergistic inhibition of 2C lysis of B10.D2 target cells by anti-CD8 mAb and 1B2 Fab fragments. (*a*) Inhibition of 2C lysis of B10.D2 target cells by varying concentrations of anti-CD8 mAb (53-6.7) in the presence of a limiting dose of 1B2 Fab fragments (see Fig. 9) or with no 1B2 Fab (medium). (*b*) Inhibition of 2C lysis of B10.D2 targets by varying concentrations of 1B2 Fab fragments in the presence of a high concentration of anti-CD8 mAb (20 μg/ml of 53-6.7) or with no anti-CD8 mAb (medium). (*c*) Inhibition by 1B2 Fab fragments of lysis of B10.D2 targets by $CD8^-$ 2C cells vs. $CD8^+$ 2C cells.

$CD8^-$ subset of 2C CTL would be much more sensitive to 1B2 inhibition than the $CD8^+$ subset. This was indeed the case. Thus, although both populations of CTL caused near-equivalent lysis of L^d targets in the absence of 1B2 (Fig. 8), the $CD8^-$ subset of 2C was 30-fold more sensitive to inhibition with 1B2 than the $CD8^+$ subset (Fig. 11 *c*).

Discussion

The results of the experiments in this paper are summarized in Table 1; for simplicity the data on the response to bm3 are omitted. The experiments were designed to address two questions. First, can HI and HD responses of $CD8^+$ cells be explained in terms of differences in the avidity of T/APC interaction? Second, do CD8 molecules play a mandatory role in primary T cell responses? TCR transgenic mice have obvious advantages for addressing these questions.

Table 1. *Summary of Data on the Responsiveness of 2C Cells to L^d vs. K^{bm11} Antigens*

2C $1B2^+$ cells tested	Assay	Response to: L^d	Response to: K^{bm11}
$CD8^+$	HI MLR	+	−‡
	HD MLR	(+)*	+
	CTL activity	+	+
$CD8^-$	HI MLR	−	−
	HD MLR	+	−
	CTL activity	+	−

* Responses to L^d are high without added lymphokines, but are even higher with lymphokines.

‡ Except for a minor subset of cells expressing a very high density of CD8.

Relationship of HI and HD Responses. With regard to the first question, the finding that a monoclonal ($1B2^+$) population of unprimed $CD8^+$ 2C cells gave an HI response to one alloantigen (L^d) and an HD response to another antigen (bm11) would seem to rule out the suggestion that HI and HD responses are mediated by different lineages of T cells (7, 8). By exclusion, the data provide strong support for the notion that HI and HD responses reflect differences in the avidity of T/APC interaction (9, 10, 36). Since the genetic backgrounds of the APCs were near identical, the data probably reflect differences in the affinity of the 2C TCR for antigen, binding of the 2C TCR to L^d being stronger than to bm11. The alternative possibility is that L^d and bm11 molecules express different densities of 2C-reactive endogenous peptides (37). Although it is difficult to choose between these two possibilities, it would seem reasonable to conclude that L^d is operationally a stronger antigen than bm11. Based on studies with soluble molecules, the intrinsic affinity of the 2C TCR for L^d plus the defined peptide recognized by this receptor (24) is 2×10^6/M (38), which is quite high. Measurements on the affinity of the 2C TCR for bm11/peptide complexes are not yet available.

The notion that HI and HD responses of $CD8^+$ cells reflect differences in the avidity of T/APC interaction predicts that any measure designed to decrease the avidity of this interaction would convert an HI response to an HD response. The effects of using heterozygous vs. homozygous stimulators are consistent with this prediction. Thus, the weak HI responses of 2C cells to bm11 and bm3 antigens were much lower with heterozygous than homozygous stimulators; other workers have observed similar findings with a different line of TCR transgenic mice (36). HI responses were also heavily influenced by the CD8 density on the responding cells. Thus, for the strong L^d antigen, HI responses were appreciably lower with $CD8^{lo}$ than $CD8^{hi}$ cells and were virtually absent with $CD8^-$ cells; conversely, the negligible HI response of unseparated $CD8^+$ 2C cells to the weak bm11 antigen increased dramatically when 2C cells were fraction-

ated into a small subset of $CD8^{hi}$ cells. These findings are in accordance with the notion that CD8 molecules act as adhesion molecules (at least in part), binding of CD8 molecules to class I molecules serving to strengthen TCR contact with antigen and thereby contributing to the overall avidity of T/APC interaction (11–13, 36, 39).

The finding that subtle alterations in the avidity of T/APC interaction had a major influence in determining whether 2C cells mediated HI vs. HD responses raises the question whether these two types of responses involve different intracellular signaling pathways. This might seem quite likely because HI responses are associated with synthesis of both IL-2 and IL-2R, whereas HD responses lead only to IL-2R expression. The current view is that IL-2R expression reflects signaling through TCR/CD3 molecules whereas IL-2 production depends on combined signaling through TCR/CD3 and CD28 molecules (40, 41). Given that a number of different cell surface molecules on T cells are capable of signal transduction in defined situations (42), one might argue that the multiple receptor/ligand interactions involved in T/APC interaction lead to a series of qualitatively different signals, the combined action of these signals being required for optimal triggering of T cells. The alternative viewpoint is that the various manifestations of T cell triggering are largely a reflection of the overall strength of the TCR/CD3-mediated signal (10). Thus, weak TCR signals are sufficient to lead to IL-2R synthesis but not IL-2 production, whereas strong TCR signals induce both IL-2R and IL-2 synthesis; the role of CD28/B7 interaction in IL-2 production (40, 41) could then be attributed to spill-over of strong TCR signals, as these signals are required to initiate or amplify the CD28 signal. in the case of IL-2R expression, it is of interest that the response of $CD8^+$ 2C cells to the weak bm11 antigen led to a much slower onset of IL-2R expression than the 2C response to the strong L^d antigen. This finding implies that IL-2R expression is quantitative and correlates with the intensity of TCR-mediated signals. Whether the same applies to IL-2 production is unclear.

Role of CD8 Molecules in Primary Proliferative Responses. In the case of heterogeneous populations of cells from normal mice, it is well established that primary responses of $CD8^+$ cells are easily inhibited with anti-CD8 antibody (11). This finding is in line with the notion that most of the T cells participating in typical primary responses have a relatively low affinity for the antigen in question and hence are CD8 dependent. If the sole role of CD8 molecules is to augment the avidity of T/APC interaction (see below), one might expect primary responses of a selected population of high-affinity $CD8^+$ cells to be CD8 independent. The data on the response of 2C cells to the strong L^d antigen are consistent with this prediction, but only in part. Thus, although the $CD8^-$ subset of 2C cells responded quite well to the L^d antigen when supplemented with lymphokines, virtually no response was observed in the absence of lymphokines. Primary proliferative responses of 2C to L^d were thus at least partly CD8-independent for HD responses but strongly CD8 dependent for HI responses.

At face value this finding suggests that optimal primary responses of T cells leading to endogenous IL-2 production cannot occur in the absence of CD8. Does this mean that CD8 molecules play an obligatory role in IL-2 production? We think this is probably not the case because purified $CD8^-$ 2C cells give strong proliferative responses to cross-linked anti-TCR antibodies in the absence of added lymphokines (our unpublished data). In view of this finding one can argue that L^d is only a "semi-strong" antigen and that a stronger antigen would lead to a fully CD8-independent response. This idea is difficult to test. Nevertheless, our hunch is that CD8 molecules do not play a mandatory role in IL-2 production. The apparent CD8-dependency of HI proliferative responses simply reflects that stimulating IL-2 production by unprimed T cells requires very strong signaling and high-avidity T/APC interaction; CD8 expression on resting cells is crucial for establishing high-avidity T/APC interaction because the range of adhesion/accessory molecules on these cells is quite low (relative to activated T cells).

Role of CD8 Molecules for CTL Activity. Though crucial for optimal proliferative responses, CD8 expression was not required for 2C CTL lysis of L^d target cells. The simplest explanation for this finding is that the high levels of various adhesion molecules on activated T cells (43) diminish the importance of CD8 molecules. It has to be remembered, however, that the parameters used to measure T cell induction and the expression of effector function are quite different. Thus, one might argue that the level of signaling required for CTL activity is much less than for initiating proliferative responses of unprimed T cells. This argument is clearly valid for HI proliferative responses, i.e., where strong signaling is required for IL-2 production. As discussed earlier, however, the signaling needed for IL-2R expression is probably quite weak. Indeed, the level of signaling required for IL-2R expression may be no higher than for the expression of CTL activity. Thus, for responses to the weak bm11 antigen it is notable that $CD8^+$ 2C cells responded quite well to this antigen in terms of both HD proliferative responses and CTL activity; by contrast $CD8^-$ 2C cells were completely unresponsive to bm11 in both assays (Table 1). This correlation is surprising. In fact, bearing in mind the much lower density of adhesion molecules on resting cells than activated cells, one is faced with the interesting possibility that the signaling required for IL-2R synthesis is less than for expression of CTL activity.

The finding that 2C lysis of bm11 (and bm3) target cells was apparent only with $CD8^+$ and not $CD8^-$ cells indicates that CD8 expression is highly important for CTL activity directed to weak antigens. Thus, as for proliferative responses, one can make the case that CD8 expression on CTL is important in any situation in which CTL interaction with target cells is suboptimal. Strong support for this view is provided by the finding that 2C lysis of L^d target cells became strongly CD8 dependent when TCR contact with antigen was impaired with limiting doses of 1B2 Fab fragments. By the same token, impairing CD8 function greatly augmented the sensitivity of 2C CTL to inhibition with 1B2 antibody.

Collectively, the data in this paper suggest that, both for the induction and effector phase, the principal function of CD8 molecules is to augment TCR contact with antigen and thereby promote high-avidity T/APC interaction and strong TCR-mediated signaling (16). This view rests on the assumption that CD8 molecules act largely as adhesion molecules. As discussed earlier (see above), however, it is clear that by associating with $p56^{lck}$ CD8 molecules also contribute to signal transduction, presumably by promoting tyrosine phosphorylation of intracytoplasmic TCR/CD3-associated molecules. Interestingly, recent studies on binding of soluble L^d/peptide complexes to 2C cells have shown that TCR binding of these complexes is highly resistant to inhibition with anti-CD8 antibody (38). This finding suggests that the adhesion function of CD8 molecules may be less important than previously considered. However, one cannot ignore the evidence that impairing the intracytoplasmic association of CD8 with $p56^{lck}$ does not abolish CD8 function (19, 20). The most likely possibility therefore is that the CD8 molecule is bifunctional, with the adhesion properties and signal transduction function of CD8 molecules being equally important.

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Address correspondence to Dr. Jonathan Sprent, Department of Immunology, IMM4, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

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