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## HELPER T CELLS FOR CYTOTOXIC T LYMPHOCYTES NEED NOT BE I REGION RESTRICTED\*

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The development of procedures for the physical or functional separation of B cells and helper T cells permitted extensive studies to be performed that have elucidated the nature of cell-cell interactions that occur during the antibody response. Current models of B cell activation state that antigen-specific helper T cells are activated by recognition of antigen in the context of H-2 I region-encoded determinants on specialized antigen-presenting cells (APC)<sup>1</sup> and then deliver inducing signals to antigen-activated B cells via recognition of antigen plus I region determinants on the B cell.

In contrast, studies of cellular interactions in the cytotoxic T lymphocyte (CTL) response have been hampered by the inability to cleanly separate CTL precursors from their helper T cells (see Discussion). Nonetheless, on the basis of mostly indirect results from a variety of experimental approaches, the most prevalent model of CTL activation is one that closely parallels models of B cell activation, i.e., that I region-restricted helper T cells activated by interaction with antigen in the context of I region determinants deliver inducing signals to antigen-activated precursor CTL (1–5). In some models, the helper T cell interacts with CTL precursors via the restricted recognition of I region determinants expressed by the CTL precursor (1, 2). Experiments interpreted as supporting the role of I region-recognition events during the process of CTL induction include (a) studies demonstrating the augmentation of CTL responses to foreign H-2K and D antigens by I region-reactive T cells (5, 6), (b) studies of CTL responses to viral (2) or non-H-2 cell surface antigens (7) or hapten (8), demonstrating an influence of I region genes expressed by cells of the thymus on the response phenotype of T cells that mature therein, (c) a study demonstrating the apparent depletion, by *in vivo* negative selection procedures, of I region-restricted helper cells required for an *in vitro* secondary CTL response specific for minor histocompatibility antigens (9), and (d) the finding that Ia<sup>+</sup> non-T accessory cells are required for *in vitro* CTL responses (4, 10, 11). In addition, studies of the Ir gene defect of nonresponder strains in the CTL response of female mice to the male antigen, H-Y, have been interpreted in terms of I region-restricted helper T cells (1, 7, 12–15).

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<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cells; C, complement; CTL, cytotoxic T lymphocyte; IL-2, interleukin 2; minor-HA, minor histocompatibility antigens; MLC, mixed lymphocyte cultures; Sp Con A Sn, supernatant from Con A-activated spleen cells.

Specifically, it is thought that H-Y antigen and a product of I<sup>k</sup> (a nonresponder allele) are an incompatible combination for restricted recognition by helper T cells. Yet, none of these studies represent a clearcut direct demonstration that I region-restricted helper T cells are required for CTL responses. Here, we present results from two experimental systems that seem inconsistent with models of secondary CTL induction that require I region-restricted recognition of antigen on APC by helper T cells. In the first set of experiments, we show that (responder × nonresponder)<sub>F1</sub> purified T cells can be induced to differentiate into H-Y-specific CTL with male stimulator cells expressing nonresponder I region alleles. In the second set of experiments, we studied the requirements for restimulation of CTL specific for minor histocompatibility antigens in long-term mixed lymphocyte cultures; here, we could demonstrate no requirement for stimulation of I region-restricted helper T cells. Helper T cells appear to be required, however, because CTL clones isolated from these long-term cultures require exogenous T helper cell factors.

Finally, we show that CTL lines cloned from long-term MLC require antigen-specific stimulation as well as exogenous interleukin 2 (IL-2) for restimulation of proliferation. This phenomenon explains the mechanism whereby CTL with particular specificities are enriched by selective antigen stimulation in long-term MLC.

### Materials and Methods

*Mice.* B10.HTG mice were provided by Dr. Martin Dorf. BALB.K, BALB.B, BALB.HTG, (BALB.B × BALB.K)<sub>F1</sub>, and B10.A(5R)/SgSnJ mice were bred at the Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA. C57BL/10 SnJ (B10), B10.D2/nSnJ, B10.BR/SgSnJ, B10.A/SgSnJ, and B10.A(2R)/SgSnJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

*Cell Depletions and Purifications.* All preparative complement-mediated cell depletions reported in this paper were performed by incubating the cells at a cell density of  $1 \times 10^7$  cells/ml, with a dilution of ascites fluid or antiserum for 30 min on ice, centrifuging the cells, resuspending them to  $5 \times 10^6$  cells/ml in rabbit complement (C) (diluted 1/20), and incubating this for 45 min at 37°C. Cells were washed twice before culture.

To deplete Ia<sup>+</sup> adherent accessory cells, lymphoid populations were passed through nylon wool columns (16), and the nonadherent cells were treated with anti-Ia serum (ATH anti-ATL serum) plus rabbit C. The surviving cells (~100% T cells) do not respond to lipopolysaccharide and do not respond to concanavalin A (Con A) unless supplemented with accessory cells or factors (see Fig. 1 legend) (17).

T cells were depleted from spleen cell populations by treatment with a mixture of two monoclonal anti-Thy-1 reagents (13-4 [18], and T24<sup>2</sup>) plus rabbit C.

Lyt-2-positive T cells were depleted from populations of long-term cultured cells by two consecutive treatments with monoclonal anti-Lyt-2.2 ascites [AD4 (15) hybridoma, ref. 19] at a dilution of 1/30, plus rabbit C. After this treatment, debris was removed by centrifuging the cells through 10 ml of fetal calf serum (FCS).

*Priming.* (BALB.B × BALB.K)<sub>F1</sub> ♀ mice were primed  $\geq 2$  wk before MLC with  $2 \times 10^7$  irradiated (BALB.B × BALB.K)<sub>F1</sub> ♂ spleen cells intravenously. BALB.B mice were primed intraperitoneally with  $1.5 \times 10^7$  B10 spleen cells.

*Mixed Lymphocyte Cells (MLC).* CTL responses to H-Y were induced by culturing accessory cell-depleted T cells from primed mice with T cell-depleted, irradiated (1,000 rad) stimulator cells in "MLC medium" (19) (containing 5% FCS and 50  $\mu$ M 2-mercaptoethanol).  $3 \times 10^6$  responder T cells were cultured with  $5 \times 10^6$  stimulators in wells of Costar 3524 plates (Costar, Data Packaging, Cambridge, MA). Cultures were assayed on day 5 for cytotoxic activity.

Long-term cultures of T cells responsive to minor-histocompatibility antigens (minor-HA) were established as described (19). The detailed history of these cultures is depicted in Fig. 2. Briefly, BALB.B spleen cells from mice primed with B10 spleen cells were boosted in bulk

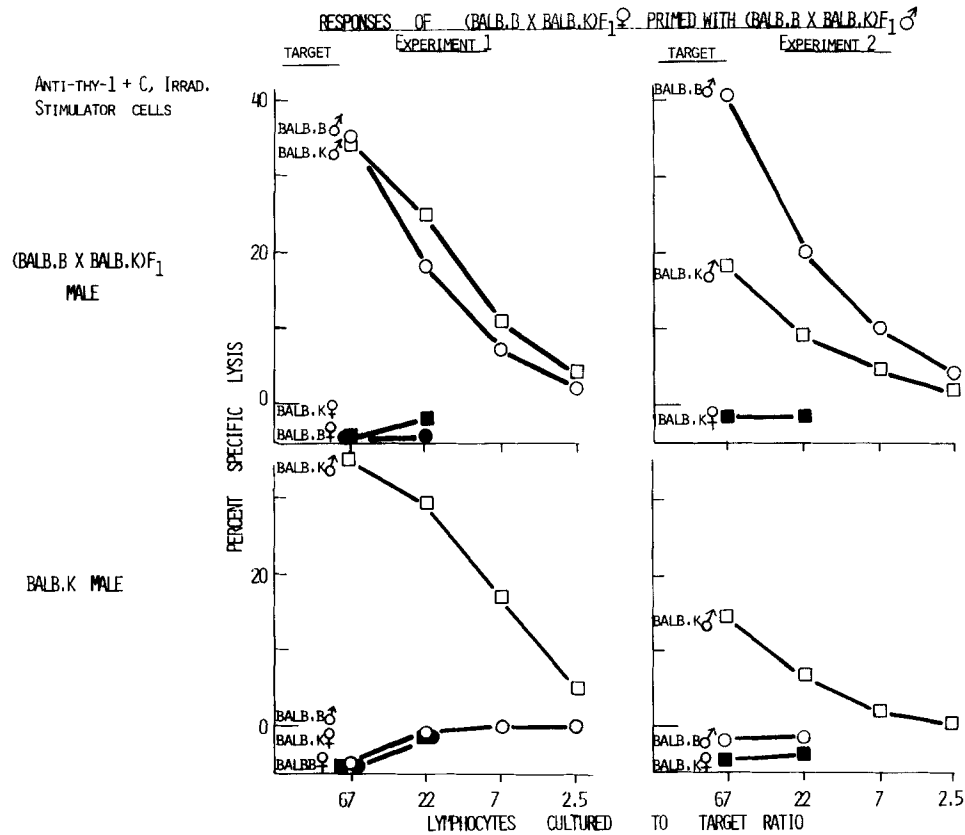


FIG. 1. Nonresponder ( $H-2^k$ ) male stimulator cells stimulate a secondary anti-H-Y CTL response. T cells purified from pooled spleen plus lymph node cells (experiment 1) or from lymph node cells (experiment 2) from primed (BALB.B X BALB.K)<sub>F1</sub> ♀ mice were boosted in vitro with the indicated T cell-depleted stimulator cells for 5 d and assayed for cytotoxic activity against the indicated Con A blast targets. As a measure of accessory cell depletion, T cells (in cultures of  $1 \times 10^5$  cells) were tested for Con A-induced mitogenesis in the presence or absence of Sp Con A Sn, with the following results (cpm [ $^3H$ ]thymidine incorporated per culture). Experiment 1: no Con A, 237; Con A, 406; Con A + Sp Con A Sn, 14,895. Experiment 2: no Con A, 40; Con A, 104; Con A + Sp Con A Sn, 5,476.

MLC ( $2.5 \times 10^7$  primed spleen cells plus  $2.5 \times 10^7$  irradiated B10 spleen cells in 20 ml of medium in Falcon 3013 flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). The cells were harvested at day 10 of culture, and  $5 \times 10^6$  recovered cells were restimulated with  $2.5 \times 10^7$  stimulator spleen cells, as above. The cells were restimulated about weekly in this fashion. The long-term culture of Lyt-2-negative cells was maintained by weekly stimulation of  $1 \times 10^6$  cells with  $2.5 \times 10^7$  irradiated B10 spleen cells in Falcon 3013 flasks.

**CTL Lines.** Cells from long-term MLC that had been enriched for  $D^b$ -restricted cells by stimulation with B10.A(2R) cells were seeded in Costar 3596 wells at 0.5, 1, or 10 cells per well (see Results), with  $5 \times 10^5$  irradiated (1,000 rad) B10.A(2R) spleen cells in 0.1 ml of MLC medium supplemented with 25–50% supernatant from spleen cells activated with Con A (Sp Con A Sn) and 25 mM  $\alpha$ -methyl mannoside. (Sp Con A Sn was prepared as described (20) from either BALB/c, BALB.K, or BALB.B spleen cells.) After 7 d at 37°C in an atmosphere of 5% CO<sub>2</sub> in air, 0.1 ml of medium, containing  $5 \times 10^5$  fresh irradiated B10.A(2R) spleen cells and 50% Sp Con A Sn was added to each well. 1 wk later, the wells were scored for cell growth, and the cells from positive wells were expanded in Costar 3524 wells with  $5 \times 10^6$  irradiated B10.A(2R) stimulator cells and 25% Sp Con A Sn, 25 mM  $\alpha$ -methyl mannoside in 2 ml of



TABLE I  
*Lyt-Phenotype of Cells from Long-Term MLC*

Cells	Antiserum treatment	Percent <sup>51</sup> Cr release ± SE*	Calculated percent of cells‡
BALB.B	C only	17.5 ± 0.5	0
Spleen	Anti-Lyt-2 + C	26.5 ± 0.5	15.4
Cells	Anti-Thy-1 + C	46.5 ± 0.6	49.6
	Anti-H-2 <sup>b</sup> + C	76.0 ± 2.0	100.0
BALB.B anti-B10	C only	10.5 ± 0.3	0
Long-term MLC cells	Anti-Lyt-2 + C	76.0 ± 0.5	95.6
	Anti-Thy-1 + C	79.0 ± 1.4	100.0
	Anti-H-2 <sup>b</sup> + C	79.0 ± 2.0	100.0

\* Percent <sup>51</sup>Cr release =  $100 \times ([\text{supernatant cpm}] / [\text{supernatant cpm} + \text{pellet cpm}])$ .

‡ Percent of cells =  $100 \times ([\text{percent } ^{51}\text{Cr release with antiserum plus C} - \text{percent } ^{51}\text{Cr release with C alone}] / [\text{percent } ^{51}\text{Cr release with anti-H-2 plus C} - \text{percent } ^{51}\text{Cr release with C alone}])$ .

MLC medium. The cell lines were maintained by weekly transfer of  $1 \times 10^5$  to  $5 \times 10^5$  viable cells to Costar 3524 wells with Sp Con A Sn plus  $\alpha$ -methyl mannoside and stimulator cells, as above, or transfer of  $1 \times 10^6$  cells to Falcon 3013 flasks with  $2.5 \times 10^7$  stimulator cells in 15 ml of medium (25% Sp Con A Sn). Normally, the cells reached a maximum cell density of  $1-2 \times 10^6$  cells/ml within 3-4 d.

*Proliferation Assays.*  $1 \times 10^5$  cells from long-term MLC were incubated with or without  $5 \times 10^5$  T cell-depleted, irradiated (3,000 rad) spleen cells in wells of Costar 3596 plates in 0.2 ml MLC medium for 3 d, at which time the cultures were pulsed for 4 h with  $2 \mu\text{Ci}$  [<sup>3</sup>H]thymidine (1 Ci/mmol) and harvested with an automated sample harvester.

Proliferation of cloned CTL was assayed as was the proliferation of cells from long-term MLC, except some of the cultures were supplemented with 25% Sp Con A Sn plus 25 mM  $\alpha$ -methyl mannoside or with a supernatant of Con A-activated AOFS-21.10.9 cells plus  $\alpha$ -methyl mannoside. AOFS-21.10.9 is an IL-2-producing T cell hybridoma line, kindly provided by Dr. J. Kappler and Dr. P. Murrack, National Jewish Hospital, Denver, CO.

*CTL Assay.* The 4-h <sup>51</sup>Cr-release assay was performed exactly as described (19), with Con A-activated spleen cells as target cells.

*Antiserum Typing of Cell Populations.* The proportions of cells in different lymphocyte subclasses were determined in a two-step antibody-dependent, complement-mediated <sup>51</sup>Cr release assay as described (19). All antibody preparations were titrated, and the values shown in Table I correspond to the levels of <sup>51</sup>Cr release with a saturating concentration of antibodies. The different antibody preparations used were: anti-Lyt-2.2, AD4(15) ascites (19); anti-Thy-1, a mixture of 13-4-1 ascites (18) and T24 culture supernatant;<sup>2</sup> and anti-H-2<sup>b</sup> serum, (C3H  $\times$  DBA/2)F<sub>1</sub> anti-BALB.B antiserum. The specificities of these reagents were confirmed in separate tests (19, or not shown).

*IL-2 Production and Assay.* Aliquots of  $3 \times 10^5$  Lyt-2-negative T cells isolated from long-term MLC were cultured in Costar 3524 wells in 1 ml of MLC medium containing  $5 \times 10^6$  T cell-depleted, irradiated (1,000 rad) stimulator cells for 36 h at 37°C. The cultures were harvested, the cells removed by centrifugation, and the supernatants sterilized by filtration. The assay IL-2 (20) dilutions of the supernatants were incubated with  $1 \times 10^4$  clone-3 cells in wells of Costar 3596 plates in 0.2 ml MLC medium supplemented with 25 mM  $\alpha$ -methyl mannoside and 10% FCS for 20 h. At that time, each culture was pulsed with  $2 \mu\text{Ci}$  (1 Ci/mmol) [<sup>3</sup>H]thymidine for 4 h before harvesting. Clone 3 is a continuously growing, IL-2-dependent, cloned CTL line from a CBA mouse that was isolated in this laboratory.

<sup>2</sup> T24 is a rat/mouse hybridoma secreting antibodies specific for mouse Thy-1 antigen and was provided by Dr. Ian Trowbridge.

## Results

*APC of the Nonresponder Haplotype Stimulate H-Y-specific CTL Responses.* The prevalent view concerning the Ir gene defect in H-2<sup>k</sup> mice, which prevents the CTL response to H-Y antigen, is that I<sup>k</sup>-encoded molecules and H-Y antigen are incompatible partners for recognition by I region-restricted helper T cells. A prediction from this model is that H-Y-specific helper T cells from (responder × nonresponder)<sub>F1</sub> mice will be activated if H-Y antigen is presented by APC of the responder but not nonresponder H-2 haplotype; if this is so, then the capacity of primed (H-2<sup>b</sup> × H-2<sup>k</sup>)<sub>F1</sub> T cells to mount a secondary H-Y-specific CTL response should depend upon the presence of APC that express I<sup>b</sup> (responder-type) gene products. To test this prediction, we depleted Ia<sup>+</sup> accessory cells (which include "conventional" APC) from T cell populations obtained from H-Y-primed (BALB.B × BALB.K)<sub>F1</sub> ♀ mice and stimulated these T cells in vitro with (BALB.B × BALB.K)<sub>F1</sub> ♂ (responder type) stimulator cells or with BALB.K ♂ (nonresponder-type) stimulator cells. Accessory cells were depleted by adherence to nylon wool, followed by treatment with anti-Ia serum and C; this procedure depletes accessory cells from T cell populations to the extent that even the Con A proliferative response is abolished, unless the cells are supplemented with accessory cells or factors (see Fig. 1, legend). To prevent the occurrence of allogeneic effects, stimulator cell populations were depleted of T cells before irradiation. As shown in Fig. 1, BALB.K ♂ stimulator cells were as efficient in boosting a CTL response directed at BALB.K ♂ targets as were (BALB.B × BALB.K)<sub>F1</sub> ♂ stimulator cells. This was true even if the source of responding T cells was lymph node cells (experiment 2); because this cell population is deficient in the stem cells from which lymphoid cells differentiate (21), (BALB.B × BALB.K)<sub>F1</sub> accessory cells cannot reappear during the 5-d culture period. We conclude that there is no requirement for presentation of H-Y antigen in the context of I<sup>b</sup> on Ia-positive accessory cells for the in vitro induction of H-Y-specific CTL.

*Anti-Minor-HA Long-Term MLC.* If I region-restricted helper T cells are involved in H-2-restricted CTL responses, there should be a requirement in secondary responses for I region matching between the APC used in the primary and secondary immunizations. Under normal circumstances, an animal is primed in vivo and, thus, the APC used in primary immunization are those of the host. An experimental determination of whether I region-mismatched stimulator cells are effective in inducing secondary responses in vitro would thus require stimulation with cells that express allogeneic I region products; however, because this protocol introduces allogeneic reactions into the system, it cannot be used to fairly assess this question.

To circumvent this problem, we chose to generate long-term cultures of cells specific for minor histocompatibility antigens (minor-HA). We reasoned that after several in vitro restimulations with stimulator cells expressing the syngeneic H-2 haplotype (but foreign minor-H antigens), alloreactive cells should be lost from the cultures because they are not stimulated. That this is so is shown in Fig. 3. Spleen cells from BALB.B mice primed in vivo with B10 spleen cells were restimulated in vitro 4 times (about weekly) with B10 spleen cells (see Fig. 2 for the history of the long-term MLC). 10 d after the fourth boost, cells were harvested from these cultures and tested for the capacity to proliferate in response to various (T cell depleted-) stimulator cells. As shown in Fig. 3, experiments 1 and 2, stimulation with B10 (homologous) stimulator cells induced high levels of [<sup>3</sup>H]thymidine incorporation in the responding cells,

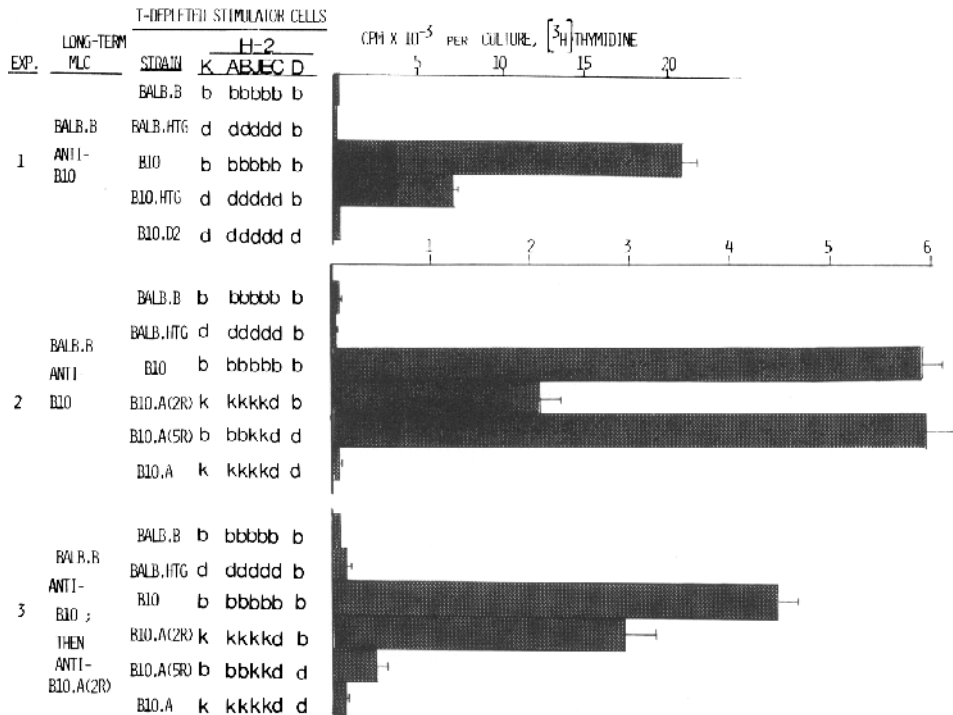


FIG. 3. H-2-restricted T cells from long-term MLC can be restimulated with stimulator cells that are matched only at the D region of H-2. 8 d after the last stimulation, cells from long-term MLC that had been stimulated four times with B10 cells (experiment 1), five times with B10 cells (experiment 2), or three times with B10 cells, followed by two stimulations with B10.A(2R) cells (experiment 3), were cultured for 3 d with T cell-depleted, 3,000 rad stimulator cells.

whereas stimulation with allogeneic cells (B10.D2 in experiment 1 and B10.A in experiment 2) induced no proliferative response above that induced using syngeneic (BALB.B) stimulator cells. From these data we make the following conclusions: (a) multiple stimulations with B10, cells responding to allogeneic H-2 antigens (i.e., H-2<sup>d</sup>, H-2<sup>k</sup>) are functionally depleted from the responding population, and (b) the proliferative response is apparently specific for B10 minor-H antigens and is restricted to H-2<sup>b</sup> antigens (because B10 but not B10.D2 or B10.A cells effectively stimulate the response).

It is important to point out that it is unlikely that these T cells are specific for antigens coding in the *Qa* region telomeric to the *H-2* complex, the CTL response to which is H-2 unrestricted (22-25). This is because BALB.B and B10 strains appear to be matched at these genes (22, 26). It is conceivable, however, that *Qa* antigens restrict part of the response to B10 minor HA. Such *Qa*-restricted cells might be among the T cells we describe below, which recognize B10.A(2R) or B10.HTG (i.e., H-2D matched) stimulator cells. For the sake of simplicity, we will include putative *Qa*-restricted T cells under the term "D restricted."

*Are I Region-matched Stimulator Cells Required in the Proliferative Response to Minor-HA?* As shown in Fig. 3, stimulator cells mismatched at the I region but matched at the D region (B10.HTG in experiment 1, B10.A(2R) in experiment 2) induce substantial proliferative responses in this system; the level of [<sup>3</sup>H]thymidine incorpo-



rated, in comparison to the level incorporated with B10 stimulator cells, reflects the fact that only a fraction of the cells will be restricted to H-2D<sup>b</sup>. The proliferating cells appear to be specific for B10 minor-H antigens because BALB.HTG stimulators, which express the identical H-2 genes as B10.HTG, fail to stimulate proliferation. Thus, stimulation of I region-restricted T cells does not appear to be required for the stimulation of D<sup>b</sup>-restricted T cells from long-term MLC. Because there is no response to cells mismatched throughout the H-2 complex, it seems unlikely that positive allogeneic effects contribute to the response of D<sup>b</sup>-restricted cells.

*D<sup>b</sup>-restricted CTL Proliferate in Response to I Region-mismatched Stimulator Cells.* To determine whether CTL are among the cells that proliferate in response to I region-mismatched stimulator cells, cultures of cells from long-term MLC were incubated for 4 d with various stimulator cells and assayed for cytotoxicity of Con A blast targets in the 4-h <sup>51</sup>Cr release assay. As shown in Fig 4B, B10.A(2R) cells (I region-mismatched) were nearly as effective as were B10 cells at stimulating CTL specific for B10.A(2R) targets. In comparison, stimulation with B10.BR cells or BALB.B cells, which are D<sup>b</sup> negative and antigen negative, respectively, yielded CTL populations with only ~10% the relative activity. The relative yield of cells recovered from the different cultures (see Fig. 4, legend) roughly correlates with the relative cytotoxic activities, especially when it is taken into account that cultures stimulated with B10 stimulator cells have cytotoxic activity restricted to K<sup>b</sup> as well as D<sup>b</sup> (see Fig. 4a; CTL from B10-stimulated cultures are more active on B10 targets than on B10.A(2R) targets). Therefore, we view the differences in the level of CTL activity induced with the different stimulator cells as reflecting differences in the level of CTL proliferation as opposed to differences in the proportion of CTL that are mature at the time of the

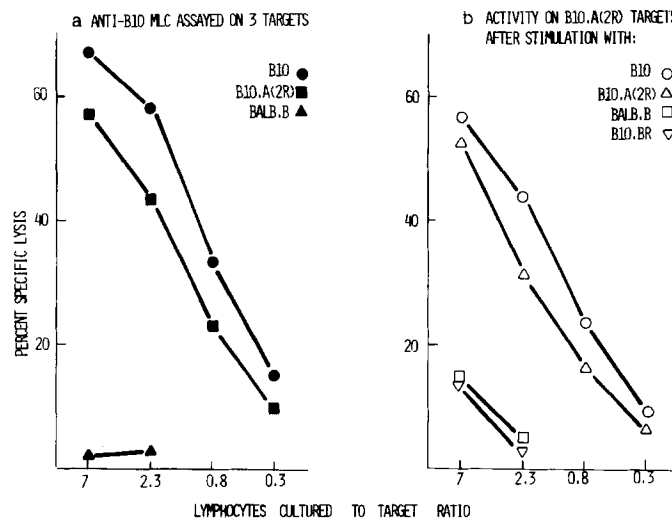


FIG. 4. Stimulator cells matched only at H-2D<sup>b</sup> restimulate minor-HA specific, D<sup>b</sup>-restricted CTL in long-term MLC.  $7 \times 10^5$  cells from long-term MLC were cultured for 4 d with T cell-depleted, irradiated stimulator cells and assayed for lysis of Con A blast targets. Numbers on the abscissa are ratios of the number of lymphocytes initially placed in culture to the number of target cells. (a) cells recovered from MLC after stimulation with B10 spleen cells were assayed on three Con A blast targets. (b) cells recovered from MLC stimulated with different spleen cells assayed for lysis of B10.A(2R) target cells. The yields of cells recovered from MLC after 4 d with the indicated stimulator cells were: B10, 280%; B10.A(2R), 155%; BALB.B, 40%; B10.BR, 58%.

CTL assay. Taken together, these results suggest that the induction of CTL proliferation does not require the participation of I region-restricted helper T cells.

**Enrichment of  $D^b$ -restricted Cells in Long-Term MLC.** To enrich  $D^b$ -restricted proliferating cells, BALB.B ANTI-B10 long-term MLC cells were restimulated in bulk MLC with B10.A(2R) cells. After two such stimulations, cells recovered from these cultures were tested for their specificity in a proliferation assay, with the results shown in Fig. 3, experiment 3. Compared to the "parent" culture (Fig. 3, experiment 2), which was maintained with B10 stimulator cells, there was a drastic reduction in the proliferative response induced with B10.A(5R) stimulator cells, whereas the response to B10.A(2R) stimulator cells was relatively increased. Thus,  $D^b$ -restricted T cells appear to have been highly enriched relative to cells restricted to  $K^b$  or  $IA^b$ . The enrichment of  $D^b$ -restricted T cells was apparent in the level of CTL activity as well. As shown in Fig. 5 b, cells from the cultures that had been selected with B10.A(2R) stimulator cells lysed B10.A(2R) but not B10.A(5R) target cells; in contrast, CTL from the cultures maintained with B10 stimulator cells lysed B10.A(5R) targets as well (Fig. 5 a).

Cells from these cultures had not been stimulated with  $I^b$ -positive stimulator cells (i.e., B10) for nearly 4 wk, and yet we could induce vigorous  $D^b$ -restricted proliferative responses. These results represent additional evidence that  $D^b$ -restricted cells can be triggered to proliferate without the contribution of I region-restricted cells.

**$I^b$ -restricted, Minor-HA-specific T Cells.** Our data, which shows that  $D^b$ -restricted, minor-HA-specific T cells from long-term MLC proliferate when stimulated with cells that are mismatched at the I region, suggests that I region-restricted helper T cells need not be involved in maintaining CTL proliferation. However, there are two alternative explanations of the data: (a) it is conceivable that  $I^b$ -restricted helper T cells in these cultures express receptors that cross-react at the level of H-2 restriction on allogeneic I region determinants (i.e.,  $I^d$ ,  $I^k$ ). This seems immediately unlikely because the receptors would have to be capable of cross-reacting on determinants of

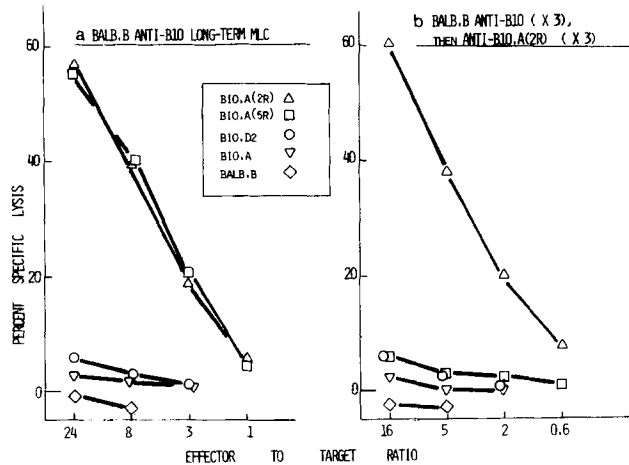


FIG. 5. Enrichment of  $D^b$ -restricted CTL specific for B10 minor-HA. See Fig. 2 for history of the MLC tested in this experiment. Cells from BALB.B anti-B10 long-term MLC (a) or BALB.B anti-B10 long-term MLC that had been further stimulated three times with B10.A(2R) stimulator cells (b) were assayed for lysis of five different Con A blast targets.

two independent allogeneic I region haplotypes; (b) it is conceivable that H-2<sup>b</sup> APC are "carried along" in the long-term MLC. These cells might be capable of processing B10 minor-H antigens from the I-mismatched stimulator cells and presenting them in the context of I<sup>b</sup> determinants on their cell surface to helper T cells in the cultures.

As shown in Figs. 3 and 4, the stimulation of T cell proliferation does require matching between the responding cells and the stimulator cells at the H-2D region, at least. This suggests that if there is antigen presentation by the putative H-2<sup>b</sup> cells, it is either inefficient or effective only for I region-restricted cells, which appear to be a minor subpopulation of the responding cells (see below). Alternatively, it could be argued that helper cells are activated to deliver helper signals but do not proliferate under these circumstances.

To evaluate the restimulation properties of I region-restricted cells, we undertook to isolate them. We found that the long-term MLC cells that survived treatment with anti-Lyt-2 plus C had the restimulation properties of I region-restricted T cells. As shown in Table I,  $\geq 95\%$  of the cells in the long-term MLC are Lyt-2-positive cells; this is a considerable enrichment in Lyt-2-positive cells compared with normal spleen cells ( $\sim 20\%$  of the T cells from BALB spleens are Lyt-2-positive; Table I, ref. 19). When the Lyt-2-negative cells from long-term MLC (2-5% of the total) were tested in the restimulation assay, we found that stimulator cells matched only at D<sup>b</sup> [i.e., B10.A(2R)] did not induce proliferation, whereas stimulators matched at the genes at the left-hand side of the H-2 complex [K<sup>b</sup>I A<sup>b</sup>B<sup>b</sup>, B10.A(5R)] did induce proliferation

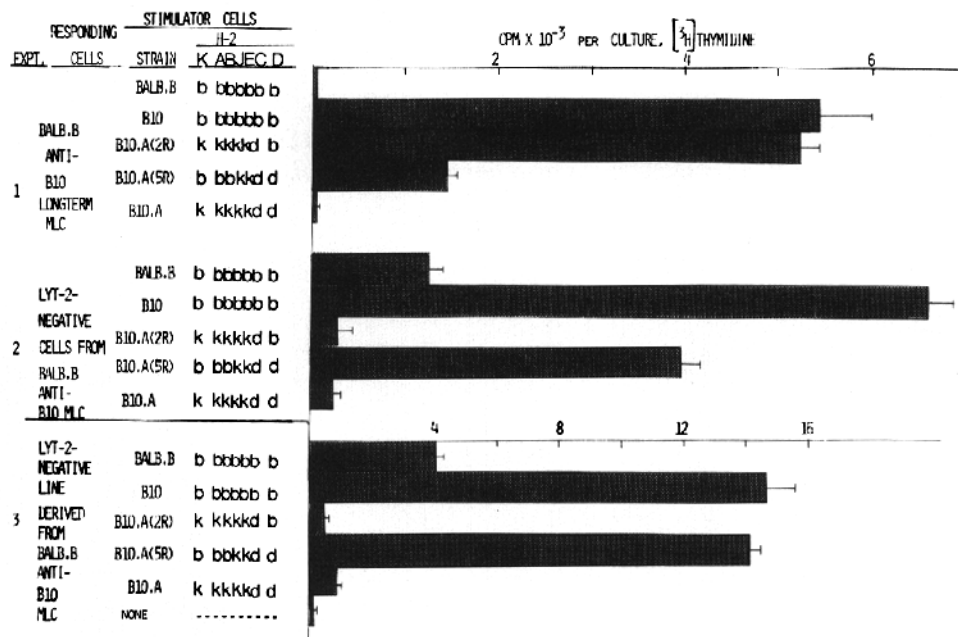


FIG. 6. Lyt-2-negative T cells from long-term MLC populations are restricted by products coding in the left end of the H-2 complex. In experiment 2, Lyt-2-negative T cells were isolated by treating long-term MLC cells twice consecutively with anti-Lyt-2 antibodies plus C; untreated cells were also tested (experiment 1). Experiment 3 shows the results with a long-term line derived by treating cells from long-term MLC with anti-Lyt-2 plus C and restimulating the surviving cells with B10 cells weekly for about 2 mo (see Fig. 2).

(Fig. 6, experiment 2). It seems likely that the Lyt-2-negative cells are restricted by IA<sup>b</sup>-encoded determinants for the following reasons: the absence of D<sup>b</sup>-restricted proliferating cells suggests that cells restricted to other class I determinants (i.e., K<sup>b</sup>) will also be lacking; furthermore, H-2<sup>b</sup> mice do not express IE/C antigens, and thus cells restricted to IE/C determinants should not be found in these cultures. Although we have not proven that these cells are I<sup>b</sup> restricted, we will refer to them as such. (The significant level of proliferation with BALB.B stimulator cells will be discussed below.)

In an independent experiment, we isolated Lyt-2-negative cells from long-term MLC populations and restimulated them weekly in further rounds of MLC with B10 stimulator cells (Fig. 2). As shown in Fig. 6, experiment 3, these cells were very similar in their restimulation properties to freshly isolated Lyt-2-negative long-term MLC cells (Fig. 6, experiment 2): B10 and B10.A(5R) cells, but not B10.A(2R) cells, stimulated high levels of proliferation. Again, this is consistent with the contention that the Lyt-2-negative cells are restricted to determinants encoded by genes in the IA<sup>b</sup> region. As was the case for freshly isolated Lyt-2-negative long-term MLC cells, a significant proliferative response was stimulated by BALB.B cells, which are syngeneic to the responding cells. We have not experimentally determined the explanation for this phenomenon, but we can suggest two possibilities: (a) a fraction of the I<sup>b</sup> region-restricted cells are specific for FCS components in the medium; these components are presented on BALB.B APC to responsive cells in the cultures. (b) B10 minor-H antigens, shed by irradiated stimulator cells in a subcellular form, are carried over to the test cultures and presented by BALB.B APC to responsive I<sup>b</sup>-restricted cells.

The results we obtained using I region-restricted cells suggest that the I<sup>b</sup>-restricted T cells do not cross-react at the level of H-2 restriction on I<sup>k</sup> (B10.A or B10.A(2R)) stimulator cells, Fig. 6., experiments 2 and 3) or on I<sup>d</sup> (B10.HTG and B10.D2; Fig. 7). Furthermore, because these stimulator cells do not stimulate the proliferation of I<sup>b</sup>-

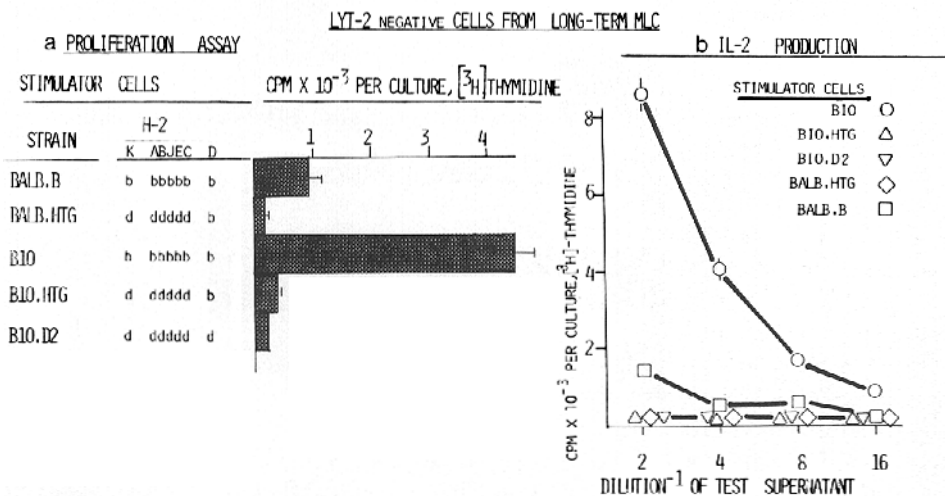


FIG. 7. Lyt-2-negative cells from long-term MLC produce IL-2 when stimulated with B10 but not B10.HTG cells. Lyt-2-negative cells, isolated from long-term MLC cells by two consecutive treatments with anti-Lyt-2 + C, were tested for (a) proliferation and (b) the production of IL-2 with various T cell-depleted stimulator cells.

restricted T cells, there does not appear to be significant numbers of functional H-2<sup>b</sup> APC carried along in the cultures.

Is it possible that I<sup>b</sup>-restricted T cells, when incubated with stimulator cells that do not express I<sup>b</sup>, are activated to deliver helper signals but not to proliferate? To determine whether the helper factor IL-2 is produced under these conditions, we isolated Lyt-2-negative cells from long-term MLC and determined their capacity to produce IL-2 after incubation with different stimulator cells. As shown in Fig. 7b, IL-2 was produced by the Lyt-2-negative cells in response to B10 stimulators, but not in response to stimulator cells that were mismatched at the left end of the H-2 complex (i.e., B10.HTG, B10.D2). This pattern of responsiveness parallels closely the pattern of the proliferative responses (Fig. 7a) and suggests that cells in these cultures will produce helper factors only if the stimulator cells express I<sup>b</sup>. Concerning these experiments, three further points should be made: (a) as will be discussed below, cloned CTL derived from long-term MLC are dependent upon IL-2-containing supernatants for growth and viability; thus, IL-2 production by cells in these cultures is a relevant helper signal to measure; (b) our similar attempts to measure production of IL-2 by unseparated populations of cells from long-term MLC failed. We suspect that this is because the IL-2 that is produced is immediately absorbed to or used by the excess of CTL in these populations. In any case, it is for this reason that we have been unable to directly determine the H-2-restriction specificity of cells that produce IL-2 in the unseparated populations; and (c) the presence of I-restricted T cells in long-term MLC populations is not evidence for the involvement of these cells in the maintenance of CTL.

*CTL Lines from Long-Term MLC Are Dependent upon Helper Factors.* We have presented data that suggests that I-restricted T cells need not be stimulated to restimulate D<sup>b</sup>-restricted CTL in long-term MLC. One possible explanation for this finding is that CTL from these cultures are helper cell independent. If this is so, then CTL clones derived from these cultures should also be helper cell independent, i.e., should replicate in the absence of added helper cells or helper factors. To assess this possibility, we derived T cell lines from long-term MLC that had been stimulated with B10.A(2R) cells (Fig. 2). The lines were derived by limiting dilution in microwells containing B10.A(2R) stimulator cells and spleen Con A Sn (see Materials and Methods); in wells that contained no Sp Con A Sn, no cell growth was observed. From a plate (96 wells) that had been seeded with 0.5 MLC cells per well, growth was observed in only a single well. Cell growth was observed in ~50% of the wells from plates that received 10 cells per well. Of these, 20 cell lines were studied further; C/DC4 and C/DC2 (Fig. 8) are examples. C/DC2H1 is a subclone of C/DC2 (subcloned at 1 cell/well; 20% cloning efficiency). In independent experiments, we achieved an ~20% cloning efficiency with cells from long-term MLC. All lines tested were found to have specific cytotoxic activity against B10 or B10.A(2R) target cells (data not shown). The lines represented in Fig. 8 were tested further and shown to lyse B10 and B10.A(2R) targets but not B10.A(5R), B10.A, or BALB.B targets (not shown); the cells thus appear to be D<sup>b</sup> restricted and specific for B10 minor-HA.

Are CTL from long-term MLC helper cell independent? It can be seen from the data in Fig. 8 that unlike the cultures from which they were derived, the CTL lines were absolutely dependent upon factors in Sp Con A Sn for growth: in the presence of B10 stimulator cells, the cells proliferated only when Sp Con A Sn was added. This

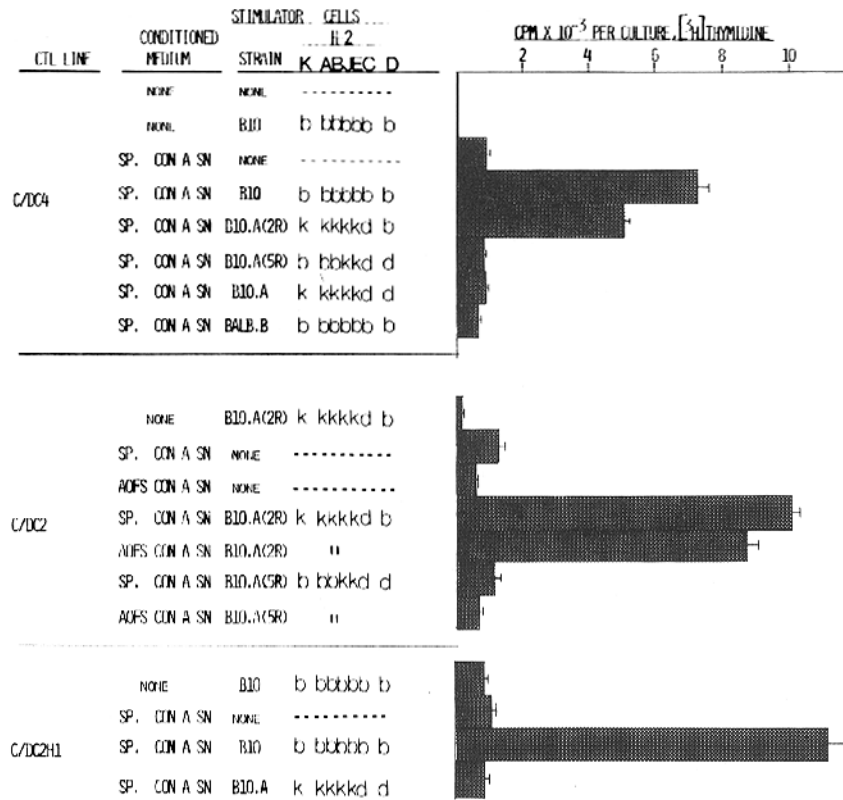


FIG. 8. Proliferation of cloned CTL lines derived from long-term MLC requires helper factors and also specific antigen. Cultures of CTL were tested for thymidine incorporation on day 3 with the indicated additions. Stimulator cells were anti-Thy-1-treated and irradiated spleen cells. AOFS Con A Sn is the IL-2-containing supernatant of Con A-activated T cell hybridoma cells.

was true for all lines tested. The relevant factors in Sp Con A Sn are probably T cell derived because supernatants from Con A-activated T cell hybridoma cells that secrete IL-2 (the AOFS-21.10.9 line provided by Dr. J. Kappler and Dr. P. Marrack) are equally capable of supporting the proliferation of these CTL lines (Fig. 8., clone C/DC2).

Based on our findings that CTL clones from these long-term MLC are helper factor dependent, whereas the parent cultures are not, we conclude that CTL in long-term MLC are helper cell dependent, and thus helper cells are present in long-term MLC populations. Therefore, our inability to detect a requirement for stimulation of I region-restricted T cells in long-term MLC does not reflect the lack of a requirement for helper cells; rather, our findings suggest that the relevant helper cells can have a different H-2 restriction specificity.

*Antigen Is Required to Induce the Proliferation of Cloned CTL.* As can be seen from the data in Fig. 8, CTL clones proliferate most efficiently when stimulated with cells that express the correct minor-HA and the appropriate H-2 antigen (i.e., D<sup>b</sup>). Thus, in our hands, syngeneic "filler" cells have no measurable effect. We conclude that the recognition of antigen by receptors on mature CTL is involved in the growth of these CTL lines. This property thus distinguishes these CTL lines from CTL lines that

grow continuously in Sp Con A Sn alone (15) and those that require syngeneic filler cells plus Sp Con A Sn (38).

### Discussion

The involvement of soluble helper factors in CTL induction was suggested in several experiments that demonstrated the augmentation of suboptimal *in vitro* CTL responses by factors in supernatants from Sp Con A Sn or MLC supernatants (3, 4, 27–32). The absolute requirement of most cloned CTL for exogenous IL-2 (15, 20, 33) that is produced by T cells (34–36) argues that a discrete helper T cell is required and that at least one function of this cell is to provide IL-2 as well as possibly other soluble mediators to CTL.

Cantor and Boyse (6) first demonstrated cellular synergy in the CTL response to foreign H-2 antigens, between Lyt-1<sup>-</sup>2<sup>+</sup> precursor CTL and Lyt-1<sup>+</sup>2<sup>-</sup> amplifier T cells. The amplifier T cells appeared to respond preferentially to foreign H-2 I region-encoded antigens, whereas the CTL were specific for H-2K and D antigens; these experiments thus extended the earlier observations of Bach et al. (5) that an ongoing response to foreign I region determinants augmented the CTL response to foreign K or D antigens. These experiments and others involving responses to foreign H-2 antigens (3) have been interpreted as evidence for an intrinsic I region specificity of helper T cells in CTL responses, although literally they show only that among alloreactive T cells there is a preponderance of I region-specific rather than K, D region-specific cells that produce augmenting signals for CTL. Allospecific CTL responses may not require the participation of T cells with specificity for I region determinants because they can be raised against cells that are allogeneic only at the D or K region as well as against cells expressing mutant K antigens (37–39).

Fitch and co-workers (33) have subsequently demonstrated cellular cooperation between cloned CTL lines and cloned amplifier T cell lines. The amplifier cell lines in these experiments were not required to interact directly with the CTL surface and were specific for M1s determinants. The role of M1s as a restricting determinant in CTL responses to non-H-2 antigens seems unlikely, however, because no M1s-restricted T cell responses have yet been demonstrated. Interestingly, a noncytolytic T cell clone that proliferates in response to foreign I region determinants did not cooperate with cloned CTL *in vitro* (38).

Evidence for I region-restricted recognition during the induction of CTL responses to non-H-2 antigens has for the most part been indirect. Zinkernagel et al. (2) and von Boehmer and co-workers (7) reported that I region antigens expressed by the radioresistant portion of the host in a bone marrow chimera or the thymus in a thymus chimera influence the ability of the T cells that mature therein to mount a CTL response. This is thought to reflect an effect of the thymus upon the selection of the I region-restriction specificity of helper T cells required for the CTL response. The conclusions of these experiments, the interpretation of which required the authors to postulate I region-restricted recognition of precursor CTL by helper T cells, have been weakened by recent experiments (40) that suggest that there is no thymus-influenced H-2 restricted recognition of precursor CTL by other T cells. Furthermore, primary CTL responses *in vivo* can occur even when (tolerant) T cells are stimulated in a completely allogeneic environment, suggesting that there is no requirement for H-2 matching between APC and CTL precursors in this response (41).

Korngold and Sprent (9), using an *in vivo* negative selection system, found that the injection of antigen-bearing cells, matched with the primed host only at the I region genes, rendered circulating host lymphocytes unresponsive in secondary *in vitro* CTL responses. That this represents selective recruitment of I region-restricted helper cells out of the circulation was not demonstrated, however.

We chose to study the H-Y system because it has provided a great deal of the evidence suggesting that helper cells in CTL responses are I region restricted. This evidence relies upon the observation that I region genes influence CTL responses: cells from  $I^b$  homozygotes but not  $I^k$  homozygotes make H-Y-specific secondary CTL responses when primed intravenously or intraperitoneally (12-15). Von Boehmer and Haas (1) demonstrated cellular synergy between cell populations from different radiation chimeras during *in vivo* priming to H-Y antigen, and their data was consistent with a requirement for  $I^b$ -restricted helper T cells for activation of  $(H-2^b \times H-2^k)F_1$  CTL specific for H-Y. The interpretation of these results has been called into question by more recent results (42) that show that nonresponder (CBA,  $H-2^k$ ) female mice respond to H-Y when primed in the footpads; the latter experiments cast some doubt on the view that  $I^b$ -restricted helper T cells are required for the H-Y response.

Our data in the H-Y system suggest that  $I^b$ -restricted helper cells are not required for secondary responses of  $(H-2^b \times H-2^k)F_1$  CTL to H-Y. Thus, we found no requirement for the stimulation of  $I^b$ -restricted T cells in this response. These results suggest that the basis of H-2-controlled nonresponsiveness to H-Y might have a different explanation than has been previously considered, and might not reflect the involvement of I-restricted cells in CTL induction. Our experiments have not established the specificity of helper cells in the H-Y response, however, and it is thus still possible that  $I^k$ -restricted helpers contribute to the response we measure. To assess a requirement for I-restricted helper cells in more detail, we turned to the minor-HA system.

In our studies of the stimulation of CTL in long-term anti-minor HA MLC, we found that antigen-bearing cells mismatched throughout the I region could stimulate the proliferation of  $D^b$ -restricted cells, including CTL. The accompanying experiments suggest that this is not because of the presentation of antigen to  $I^b$ -restricted helper cells by contaminating  $H-2^b$  APC, nor to cross-reactivity of receptors of  $I^b$ -restricted helper T cells, nor to positive allogeneic effects. Thus, I-restricted helper cells are not required to support CTL growth under these conditions. Yet, the fact that none of 14 CTL lines cloned from the long-term MLC would proliferate without an exogenous source of IL-2 argues that a discrete class of IL-2-producing cells is required for CTL growth and is present in the MLC. Because stimulator cells matched only at D are sufficient to boost the long-term MLC, the IL-2-producing cells are likely to be K/D-restricted cells; furthermore, they appear to be  $Lyt-2^+$  cells because no D-restricted cells were found among the small numbers of  $Lyt-2^-$  cells present in the cultures.

Possibly because of their small numbers, we were unable to find D-restricted IL-2-producing cells among our cloned lines from the long-term MLC; furthermore, in cloning experiments in which spleen Con A Sn was omitted from the culture medium, no cell growth was observed in any of the wells. Thus, we are unable to directly characterize these cells. *A priori*, they might represent a class of D-restricted noncytotoxic "helper cells for CTL." Alternatively, a subpopulation of CTL capable of



producing IL-2 might support the growth of other CTL in the cultures. The existence of such factor-producing CTL, which do not require exogenous IL-2, was recently reported by Widmer and Bach (43), who found that one of seven cloned CTL lines tested had this capability. Thus, IL-2-producing CTL might serve as helper cells for CTL that do not produce factors.

Whatever the correct explanation, we found no demonstrable requirements for I-restricted helper cells in the secondary CTL response. However, our findings do not rule out a requirement for I-restricted cells in the primary response. Nonetheless, from a theoretical point of view, it is worth considering that a requirement for two cells restricted to different H-2 antigens for a particular immune response subjects the animal to "double jeopardy" in terms of the potential for H-2-controlled low responsiveness. From this perspective, we find it appealing to consider that no I-restricted cell is required for CTL induction.

Finally, in agreement with others (44-46), we find that CTL clones require H-2-restricted antigen recognition to proliferate even in the presence of Sp Con A Sn. Thus, activated CTL do not necessarily proliferate indefinitely in the presence of IL-2. This finding is at variance with reports that primed CTL or activated CTL require only nonspecific factors (i.e., IL-2) for restimulation. Those lines of CTL that grow continuously in media containing IL-2 but not stimulator cells are most probably "pseudo-transformed" variants; this conclusion is supported by a report that heterogeneous populations of CTL from MLC are rendered homogeneous in specificity (presumably clonal) after adaptation to growth in media containing IL-2 but no stimulator cells (47). In any case, the finding that even cloned CTL require specific antigen stimulation provides at least a partial explanation for the observation that activated CTL with a particular specificity can be enriched in long-term MLC by selective restimulation with antigen (e.g., Fig. 5, and ref. 48). Further analysis of the accessory cell and helper T cell requirements of CTL should help to further define the mechanism of CTL induction.

### Summary

We investigated the antigenic requirements for restimulation of H-2-restricted cytolytic T lymphocytes (CTL) *in vitro* to determine whether H-2 *I* region-restricted helper T cells are required in these responses. In one set of experiments, we studied the *in vitro* response of (responder  $\times$  nonresponder) $F_1$  female T cells to the male antigen H-Y. We chose to examine this response because it has been suggested that the defect in nonresponder strains is a failure of helper T cells to recognize H-Y in association with nonresponder *I* region determinants. However, we find that nonresponder male stimulator cells are as effective as  $F_1$  male stimulator cells at inducing H-Y-specific CTL responses. This finding calls into question reports that secondary CTL responses to H-Y are dependent upon the activation of H-Y-specific helper T cells restricted to responder type *I* region determinants.

In a second set of experiments, we examined the requirements for restimulation of H-2-restricted T cells specific for minor-histocompatibility antigens from long-term mixed lymphocyte cultures. These cultures were established by repeatedly restimulating cultures of specific T cells with H-2-matched stimulator cells expressing foreign minor histocompatibility antigens. We found that H-2D-restricted T cells, including CTL, could be restimulated with cells that were matched with the responding cells at

only the D region genes. This response did not appear to result from positive allogeneic effects or from antigen processing and "representation" by responder type APC that might contaminate the cultures. Thus, we find no evidence for a requirement for I region-restricted helper T cells in these CTL responses. However, helper T cells are required because we find that CTL lines derived by limit-dilution cloning from these long-term MLC are absolutely dependent upon exogenous helper factors for growth. The most simple interpretation of these results is that the helper cells are restricted to H-2 antigens other than I region antigens or to antigens that code outside of the H-2 complex.

Finally, we show that factor-dependent CTL lines must recognize their specific antigen to proliferate, even in the presence of exogenous factors. The requirement of activated CTL for antigen to proliferate provides an explanation for how specific CTL can be selectively enriched in MLC by specific antigen stimulation. Furthermore, it is at variance with reports that memory CTL or activated CTL require only interleukin 2 for restimulation.

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