

# MULTIPLE PATHWAYS OF T-T INTERACTION IN THE GENERATION OF CYTOTOXIC T LYMPHOCYTES TO ALLOANTIGENS\*

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T helper (Th)<sup>1</sup> cells participate in the generation of cytotoxic T lymphocytes (CTL) to alloantigens (1–5). Soluble factors can substitute for Th cells in activating unprimed (6–9) or primed (10) CTL. However, little is known about the mechanisms that regulate the interaction of Th cells with CTL precursors (CTL.P). Pilarski (5) reported that the interaction between unprimed peripheral Th cells and CTL.P from the thymus occurred between allogeneic as well as syngeneic T cells, as long as both could respond to stimulator alloantigens. These results were taken to indicate that T-T interaction in the generation of CTL is not restricted by determinants encoded within the major histocompatibility complex (MHC). An alternate interpretation is that Th cells with restricted activity for CTL.P do exist, but their activity is masked by allogeneic effects. Their presence may be revealed only after clonal expansion and selection during priming.

Therefore, we have investigated the nature of Th-CTL.P interactions with both unprimed and primed populations of Th cells. We report here that unprimed Th cells show no apparent restriction in their interactions with CTL.P, which confirms the results of Pilarski (5). However, Th cells can mediate potent allogeneic effects in response to determinants on CTL.P, thereby providing help for allogeneic thymocyte populations. When such allogeneic effects are minimized, primed populations of Th cells are restricted in their activity, interacting preferentially with CTL.P that express self-markers encoded by MHC-linked genes.

## Materials and Methods

*Mice.* C57BL/10 ScSn (B10), AKR/J, C3H/HeJ, 129/J, A.By, and B10.D2 ScSn strains were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/cCr (C) mice were purchased from Health Science Inc., West Seneca, N. Y., or bred in the colony of Dr. D. B. Amos at Duke University Medical Center (Durham, N. C.). All other mice used in this study,

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<sup>1</sup> *Abbreviations used in this paper:* BSS, balanced salt solution; C, BALB/cCr; <sup>51</sup>Cr, Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>; CTL, cytotoxic T lymphocyte(s); CTL.P, CTL precursor(s); FCS, fetal calf serum; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; NaPBS, phosphate-buffered saline prepared free of Mg<sup>++</sup> and Ca<sup>++</sup> salts; Th, T helper.

including C57BL/6J (B6), C3H/St, C3H.SW, A/J, A.SW, DBA/2Ha(D2), and (C57BL/6 × DBA/2)F<sub>1</sub> (B6D2F<sub>1</sub>) were bred in the colony of Dr. Amos.

*Reagents.* All reagents were prepared in glass-distilled water. Phosphate-buffered saline prepared free of Mg<sup>++</sup> and Ca<sup>++</sup> salts (NaPBS) was used. Hanks' balanced salt solution (BSS) was supplemented with 1% glucose. The hypotonic solution used for lysis of erythrocytes has been described (11). RPMI-1640 was prepared from powder (Grand Island Biological Co., Grand Island, N. Y.) and stock solutions were supplemented and buffered as previously described (11). For cell cultures, RPMI-1640 was supplemented immediately before use with fetal calf serum (FCS) (batch A671424, Grand Island Biological Co.), 2-mercaptoethanol, penicillin, streptomycin, and L-glutamine as described (12). Rabbit and guinea pig complement were purchased frozen from Pel-Freeze Biologicals Inc., Rogers, Ark.

*Preparation of Cells.* Donors of spleens and lymph nodes were used at 8–16 wk of age. Thymus donors were generally 4–6 wk of age. Organs were removed aseptically into ice-cold NaPBS supplemented with 2% FCS, and single-cell suspensions were prepared by gently pressing tissue through a fine steel mesh with a rubber plunger. Cells were washed twice before use. Erythrocytes were lysed in preparations of spleen and lymph node cells before the final wash. Lymphocytes were resuspended at the desired viable cell concentration as determined by trypan blue exclusion. Cells were irradiated with a <sup>137</sup>Cs source (Gammacell 40, Atomic Energy of Canada Ltd., Commercial Products, Ottawa, Canada; dose = 134 R/min). Stimulator cells were irradiated with 3,300 R, whereas populations assessed for Th cell activity were irradiated with 1,000 or 1,500 R. No differences in function were noted with the different irradiation doses.

Tumors that were used as target cells in cytotoxic assays were maintained in ascites form by *in vivo* passage in the following strains of mice: P815, D2; EL4, B10, or B6; RL♂ 1, C; RDM-4, AKR. RDM-4 was kindly provided by Dr. R. J. Hodes, Immunology Branch, National Cancer Institute, Bethesda, Md.

*Cell Culture.* Responding lymphocytes (mixed spleen and lymph node, 5 × 10<sup>6</sup>/ml) were primed in one-way bulk mixed lymphocyte culture (MLC) against irradiated stimulator cells (10<sup>7</sup>/ml) as previously described in detail (11, 12). Primed T cells were used 7–14 d after initiation of culture. Sequentially restimulated MLC responder cells were recultured at 5 × 10<sup>5</sup> cell/ml; otherwise, conditions of cultures were the same as those used in primary bulk MLC. Cells from these cultures were restimulated every 2 wk.

To assess helper function in unprimed (mixed spleen and lymph node) or MLC-primed lymphocytes, the cells were irradiated and various numbers (Results) were added in a volume of no more than 1 ml to flasks (No. 3013, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) that contained 3 ml (5 × 10<sup>6</sup>/ml) thymocytes and 3 ml (10<sup>7</sup>/ml) irradiated splenocytes as a source of stimulator cells. Cytotoxic activity was measured 5 d later. Populations to be used as helper cells were irradiated to eliminate their contribution to cytotoxic activity measured on day 5 (Results). Therefore, under these conditions, the helper cell activity measured is a function of radiation-resistant cells. This system is similar to that described by Pilarski (5).

*UV-Light Irradiation.* Splenocytes were resuspended in NaPBS without serum at 1–2 × 10<sup>7</sup>/ml, and 7-ml aliquots were irradiated in 100-mm Petri dishes. Cells were irradiated by 5-min exposure to a short-wave UV light (Ultra Violet Products, Inc., San Gabriel, Calif., dose = 1,200 μW/cm<sup>2</sup> at 254 nm 6 in. from the source). After irradiation, cells were washed three times before use.

*Cytotoxic Assay.* Cytotoxic activity of lymphocytes was measured against target cells labeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (<sup>51</sup>Cr) as previously described (13). Briefly, 0.25 ml of target cell suspension (25 × 10<sup>3</sup>) was mixed with 0.25 ml of lymphocytes in 10- × 75-mm tubes, centrifuged at 300 *g* for 1 min, and incubated for 3 h at 37°C. 1 ml of cold NaPBS was added at the end of the incubation period, the tubes centrifuged, and supernate collected. <sup>51</sup>Cr activity in the supernate was counted in a well-type gamma counter (model 1185, Nuclear-Chicago Corp., Des Plaines, Ill.). Percent specific <sup>51</sup>Cr released was calculated as:

$$\text{Percent specific } ^{51}\text{Cr released} = \left( \frac{E - S}{M} \right) \times 100\%$$

where E is counts per minute released in the presence of lymphocytes, S is spontaneous release, and M is maximum release caused by freeze-thaw lysis of target cells. Triplicate determinations of each test were performed. Standard error of the mean counts per minute  $^{51}\text{Cr}$  released within groups was usually 2–3%.

Two to three lymphocyte:target ratios were assayed in each experiment with one of two methods. First, lymphocytes recovered from culture were resuspended at  $10^6/\text{ml}$  and subsequently diluted such that lymphocyte:target ratios were constant in all groups at 10:1, 3:1, and 1:1. Alternatively, recovered lymphocytes were resuspended in 4 ml, and aliquots of the total culture were assessed for cytolytic activity. With this method, n, 0.33 n, and 0.1 n represent  $1/6$ ,  $1/48$ , and  $1/160$  of the culture/ $25 \times 10^3$  target cells, respectively. Cytotoxic activity measured in this manner reflects the recovery of cells from culture as well as the amount of cytotoxic activity per unit cell number.

*Preparation and Use of Anti-Thy-1 Antisera.* Anti-Thy-1.2 antiserum was prepared in AKR mice immunized with C3H/HeJ thymocytes. Anti-Thy-1.1 was prepared in C3H/HeJ mice against AKR thymocytes. For both antisera, mice were immunized intraperitoneally once every 2 wk and bled 7 d after each immunization following the third immunization. Sera were collected, and pools of anti-Thy-1 antisera were made from up to 15 immunization cycles. Pools were ultracentrifuged (12,000 g) and sterile filtered before use. Anti-Thy-1 treatment of cells consisted of a two-step procedure. Cells were resuspended in the appropriate dilution of antiserum in BSS, incubated on ice for 30 min, washed once, and incubated with complement (rabbit complement, 1:35, or guinea pig complement, 1:20) for 45 min at  $37^\circ\text{C}$ . Cells were then washed three times before use.

## Results

*Helper Activity is Provided by a T Cell.* Most primed lymphocytes recovered from MLC are Thy-1 positive (12), and it is therefore unlikely that non-T cells contribute significantly to the helper activity. The results shown in Table I confirm that the helper activity of B6 anti-C cells for a B6 thymocyte response to C stimulator cells is susceptible to treatment with anti-Thy-1.2 and complement. Similar results were obtained by Pilarski with unprimed splenocytes (5) or splenocytes cultured in MLC for 2 d (14).

*Cytotoxic Activity is Derived from CTL.P in the Thymus and Not from the Helper Population.* In these experiments it is of utmost importance to confirm that the cytotoxic response measured after 5 d of culture results from the generation of CTL in the thymocytes and does not reflect cytotoxic activity in the helper population. Although irradiation inhibits proliferation of MLC-primed cells, it does not prevent reactivation of CTL in the primed Th population (15, 16). Two types of experiments have been performed to establish the source of cytotoxic activity. In the first, the efficiency of irradiation of the Th population was routinely confirmed by including controls in which both Th cells (syngeneic and allogeneic to the thymocyte donor [see below]) and thymocytes were irradiated. In these cultures, cytotoxic activity was never detected, which suggested that CTL from cultures that contained irradiated MLC-primed Th cells and unirradiated thymocytes are derived solely from the thymus population.

With a more direct approach, C3H (Thy-1.2) or AKR (Thy-1.1) thymocytes were cultured with B10.D2 stimulator cells and irradiated anti-B10.D2-primed helper cells expressing the alternate Thy-1 allele (AKR or C3H, respectively). After 5 d the cultured cells were recovered, washed, and divided into three treatment groups; complement alone, anti-Thy-1.1 and complement, or anti-Thy-1.2 and complement. The cytotoxic activity of the treated populations is shown in Fig. 1. In the first

TABLE I  
*Helper Activity is Mediated by a T Cell\**

Number of helper cells added ( $\times 10^{-6}$ ) <sup>‡</sup>	Pretreatment of helper cells	Percent specific <sup>51</sup> Cr released effector:target ratio	
		10:1	3:1
		%	
—	—	1.6	0.1
1	C' control	9.4	3.8
1	Anti-Thy-1.2 + C'	2.1	0.8
3	C' control	19.0	9.6
3	Anti-Thy-1.2 + C'	3.1	1.8

\* Cytotoxic activity was generated in B6 thymocytes against C stimulators and measured on P815 target cells. Irradiated MLR-primed B6 anti-C cells were used as a source of helper cells.

<sup>‡</sup> MLC-primed helper cells were treated with medium and rabbit complement (C') or anti-Thy-1.2 and C' before initiation of culture. The number of helper cells added was determined in the C' controls, whereas the anti-Thy-1.2-treated helpers were equalized to the same volume. Few viable cells remained after this treatment (<7%). Note that the addition of  $1 \times 10^6$  and  $3 \times 10^6$  primed cells represents 1/15 and 1/5 the number of thymocytes in culture, respectively.

experiment (Fig. 1 A and B), only the antiserum directed against the Thy-1 antigen of the thymocyte donor was effective in eliminating cytotoxic cells. Because the treatment did not completely abolish CTL activity, a second experiment was performed with twice the concentration of anti-Thy-1 antisera to ensure that the response was not partially a result of Thy-1-negative cells (Fig. 1 C and D). The Thy-1 antiserum against the antigen of the thymocyte donor removed all cytotoxic activity, whereas the Thy-1 antiserum against the helper cell Thy-1 antigen had no consistent effect. These results demonstrate that the cytotoxic cells detected in this system are T cells derived from thymocyte precursors.

*Strain Specificity of Th-CTL.P Interaction.* The strain specificity of Th-thymocyte interaction was tested with unprimed or MLC-primed syngeneic and allogeneic lymphocytes. B6 thymocytes were cultured with AKR stimulator cells, and unprimed or anti-AKR-primed (day 10) Th cells from B6 or D2 mice were titrated into the cultures. Cytotoxic activity was measured 5 d later. As shown in Table II, the cytotoxic activity of B6 thymocytes was amplified in the presence of any of the four helper populations, albeit to differing degrees. The most active helper population was the primed syngeneic B6 anti-AKR, whereas unprimed B6 cells were the least active. This suggests that MLC priming increases the number of radioresistant helper cells.

Both primed and unprimed D2 cells provided helper activity for B6 thymocytes. In contrast to results with B6 helper cells, priming of D2 cells did not markedly increase their activity. This suggests that the helper activity provided by D2 cells may result from recognition of determinants on B6 CTL.P rather than on AKR stimulator cells. The Th activity of D2 cells may, therefore, be a result of an allogeneic effect against the effector cell precursor, not unlike that which stimulates B cell responses (12).

*Allogeneic Effects Provide Th Activity for Cytotoxic Precursors.* To directly test whether

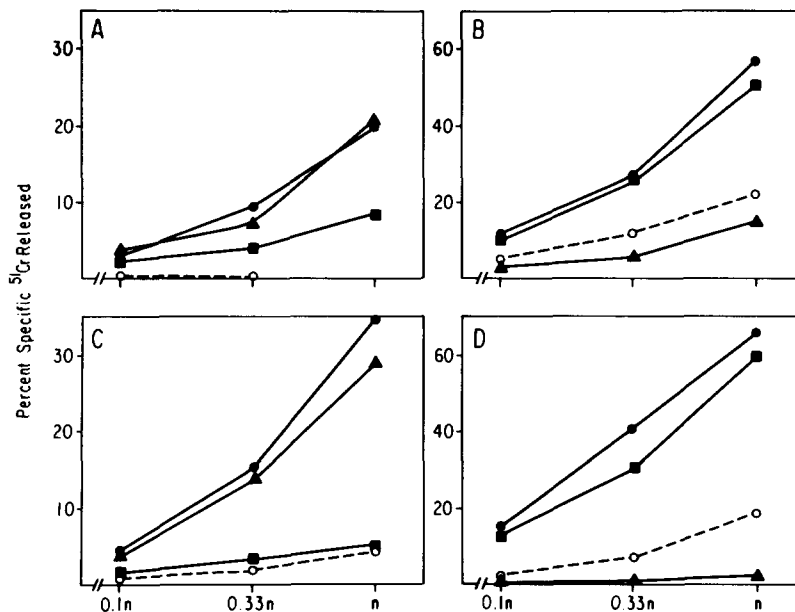


FIG. 1. Cytotoxic activity is derived from CTL.P in the thymus. C3H (A and C) or AKR (B and D) thymocytes were cultured with B10.D2 stimulators in the presence of  $3 \times 10^6$  MLC-primed AKR anti-B10.D2 or C3H anti-B10.D2 helper cells, respectively. After 5 d of culture each population was divided into three groups and treated with the appropriate anti-Thy-1 antisera and complement before measurement of cytotoxic activity on  $^{51}\text{Cr}$ -labeled P815 target cells. (O) Thymocytes and stimulator cells with no added helper cells; (●) thymocytes cultured with stimulator cells and helper cells then treated with guinea pig complement before cytotoxic activity was tested; (▲) the same combinations of cells treated with anti-Thy-1.1 and complement; (■) cells treated with anti-Thy-1.2 and complement. Cytotoxic activity was measured at different lymphocyte:target ratios as represented on the abscissa (Materials and Methods). The results of two experiments are shown. (A and B) Dilution of anti-Thy-1 antisera used was 1:10. Maximum  $^{51}\text{Cr}$  released by P815:  $3,791 \pm 69$ ; spontaneous release:  $314 \pm 16$  (8.3% of maximum). (C and D) Dilution of anti-Thy-1 antisera used was 1:5. Maximum  $^{51}\text{Cr}$  released by P815:  $6,622 \pm 18$ ; spontaneous release:  $508 \pm 19$  (7.6% of maximum).

allogeneic effects can provide helper activity for CTL.P, the following experiments were performed. First, advantage was taken of the observation that UV-irradiated allogeneic cells do not stimulate primary cytotoxic responses (17). Spleen and lymph node responder cells were mixed with UV-irradiated C3H stimulators, and various types of helper populations were titrated into the cultures. For responder cells, B6D2F<sub>1</sub> were used to limit reactivity against the helper populations (B6 or C) that could provide third-party stimulation (17). Thus, any activity provided by the helper populations should be a result of their recognition of the B6D2F<sub>1</sub> responder cells. The results are shown in Table III. All three helper populations used, including unprimed B6, MLC-primed B6 anti-C, and C anti-B6 provided helper activity for the induction of strong anti-H-2<sup>k</sup> cytotoxic responses against the UV-irradiated C3H stimulator cells. The decreased response with larger numbers of primed helper cells may reflect the activity of cells that mediate negative allogeneic effects (12).

It is possible in this experiment that MLC-priming qualitatively altered the ability of Th cells to respond to UV-irradiated stimulator cells. Therefore, the activity provided by the B6 anti-C and the C anti-B6 populations might reflect, at least in

TABLE II  
*Activation of CTL in the Thymus After Addition of Lymphocyte Populations with Helper Activity\**

Helper cells	Number added ( $\times 10^{-6}$ ) <sup>‡</sup>	Percent specific <sup>51</sup> Cr released from H-2 <sup>k</sup> targets at effector:target ratio <sup>§</sup>		
		10:1	3:1	1:1
—	0	5.1	1.8	2.5
Unprimed B6	2.0	6.6	3.1	1.6
Unprimed B6	6.0	8.0	3.6	2.3
Unprimed B6	20.0	14.0	3.0	1.1
Unprimed B6	60.0	2.2	1.7	0.2
B6 anti-AKR	0.1	5.0	3.6	0.0
B6 anti-AKR	0.3	6.1	5.0	2.3
B6 anti-AKR	1.0	27.0	15.9	7.9
B6 anti-AKR	3.0	37.0	26.5	17.2
B6 anti-AKR	3.0	37.5	23.4	17.0
Unprimed D2	0.6	5.9	1.4	0.3
Unprimed D2	2.0	10.4	4.8	3.1
Unprimed D2	6.0	15.0	7.7	3.6
Unprimed D2	20.0	23.1	12.7	8.2
D2 anti-AKR	0.1	8.1	3.6	2.1
D2 anti-AKR	0.3	8.0	3.0	3.8
D2 anti-AKR	1.0	7.5	3.1	3.2
D2 anti-AKR	3.0	19.0	10.7	5.3
D2 anti-AKR	6.0	25.6	12.9	5.2

\* CTL.P were from B6<sub>r</sub> ( $1.5 \times 10^7$ ) stimulated with  $\gamma$ -irradiated AKR stimulators ( $3 \times 10^7$ ).

<sup>‡</sup> Twice the number of unprimed helpers were added compared with primed helpers to compensate for the difference in T cells. Primed cells were from day 10 of MLC.

<sup>§</sup> RDM-4 was used as the H-2<sup>k</sup> target. Maximum <sup>51</sup>Cr released:  $2,359 \pm 93$ ; spontaneous <sup>51</sup>Cr released:  $381 \pm 32$  (16.2% of maximum). No cytotoxic activity was detected against EL4 (H-2<sup>b</sup>) targets in any combinations. Cultures that contained D2 helper populations were assayed for CTL to P815 (H-2<sup>d</sup>) targets to determine the stimulator capacity of the cells; minimal activity was detected in cultures with D2 anti-AKR, strong reactions were detected in cultures with the highest ( $20 \times 10^6$ ) number of unprimed D2.

|| Results from a duplicate culture.

part, responses of the cells to the C3H stimulator. However, this is certainly not the case with unprimed B6 helper cells. Therefore, Th activity provided by these cells must represent an allogeneic effect in the strictest sense.

In a second experiment, the same three helper populations used in the previous experiment (Table III) as well as primed B6 anti-C3H cells were tested for helper activity in cultures of B6 thymocytes and C3H stimulator cells. The data are presented in Table IV. The most active help was provided by cells primed against the thymocyte responder (C anti-B6). In comparing this population with syngeneic B6 anti-C3H and with unprimed B6 cells, in which essentially no helper activity was detected, these data suggest that allogeneic cells can be more potent sources of Th activity than syngeneic helper populations. It is also noteworthy that the B6 anti-C population was inactive in this combination, although it clearly contains active Th cells when used with other CTL.P (Table III).

TABLE III  
*Allogeneic Effects Provide Helper Activity for Cytotoxic Precursors\**

Irradiation of stimulator cells‡	Helper cell populations		Percent specific <sup>51</sup> Cr released at effector:target ratio§		
	Source	Number (× 10 <sup>-6</sup> )	10:1	3:1	1:1
			%		
γ	—	—	32.0	20.6	13.4
UV	—	—	4.9	2.8	2.6
UV	Unprimed B6	0.06	6.6	3.3	3.3
UV	Unprimed B6	0.20	11.2	6.3	2.9
UV	Unprimed B6	0.60	16.8	7.9	4.7
UV	Unprimed B6	2.00	23.3	13.5	5.9
UV	Unprimed B6	6.00	31.6	16.4	9.4
UV	B6 anti-C	0.03	3.2	3.6	2.8
UV	B6 anti-C	0.10	7.9	2.6	1.4
UV	B6 anti-C	0.30	20.4	11.4	5.4
UV	B6 anti-C	1.00	12.5	17.9	3.6
UV	B6 anti-C	3.00	7.3	3.4	2.4
UV	C anti-B6	0.03	29.5	20.5	9.9
UV	C anti-B6	0.10	38.0	26.7	15.9
UV	C anti-B6	0.30	39.8	24.0	15.4
UV	C anti-B6	1.00	33.5	32.4	10.0
UV	C anti-B6	3.00	19.2	9.3	5.9

\* Responder cells were mixtures of spleen and lymph nodes from B6D2F<sub>1</sub> mice.

‡ Stimulator cells were γ- or UV-irradiated C3H spleen cells (Materials and Methods).

§ Measured on RDM-4 (H-2<sup>b</sup>) target cells; maximum <sup>51</sup>Cr released: 7,412 ± 155; spontaneous release: 1,523 ± 65 (20.5% of maximum). No significant cytotoxic activity was detected on EL4 (H-2<sup>b</sup>) or RLδ-1 (H-2<sup>d</sup>) targets.

*Syngeneic Preference in T-T Interaction.* That allogeneic effects against CTL.P-containing responder populations can give rise to active CTL suggests that the helper activity in the D2 anti-AKR-primed population for B6 thymocytes (Table II) may be in part a result of allogeneic effects against B6 cells rather than a specific cooperative interaction between allogeneic Th and CTL.P cells in response to H-2<sup>k</sup> stimulator cells. Thus, if allogeneic effects are minimized, self-restricted interactions may be revealed.

We have found that MLC-primed cells often lose their capacity to mount allogeneic effects against thymocytes after repeated stimulation *in vitro*. One such restimulated population, a B10 anti-C combination, displayed a decreased ability to mediate allogeneic effects on C thymocytes even though it still proliferated actively in response to C stimulators and retained the ability to help B6 thymocytes in CTL responses to C stimulators. These lymphocytes were used to determine whether T-T interaction is self restricted. The restimulated B10 anti-C population was therefore irradiated and used as a source of Th cells for thymocytes from B6, C3H, A, and A.SW mice in an anti-C cytotoxic response. The results are presented in Table V. The B10 anti-C helper population was strongly active with B6 thymocytes but minimally active with CTL.P from other donors. It is possible that this latter activity was a result of weak allogeneic effects still exerted by the helper cells as revealed on C thymocytes in anti-C3H responses. It should be noted that the failure of C3H, A, and A.SW thymocytes

TABLE IV  
*Helper Activity of Syngeneic and Allogeneic Lymphocytes with Thymocytes as a Source of CTL Precursors\**

Helper cells		Percent specific <sup>51</sup> Cr released at effector:target ratio‡		
Source	Number (× 10 <sup>-6</sup> )	10:1	3:1	1:1
—	—	1.3	0.9	0.6
Unprimed B6	2.0	3.7	2.3	2.3
Unprimed B6	6.0	2.7	0.9	2.2
Unprimed B6	20.0	3.3	1.9	0.9
B6 anti-C3H	1.0	3.8	0.0	1.2
B6 anti-C3H	3.0	4.9	2.5	1.0
B6 anti-C3H	10.0	11.0	6.0	3.2
B6 anti-C	1.0	3.5	1.0	0.7
B6 anti-C	3.0	3.3	1.3	1.3
B6 anti-C	10.0	4.7	3.1	1.3
C anti-B6	0.1	7.4	3.8	2.5
C anti-B6	0.3	19.7	11.3	5.9
C anti-B6	1.0	47.2	26.6	11.9
C anti-B6	3.0	7.4	3.6	2.2

\* CTL precursors were from B6<sub>T</sub> cultured with C3H stimulators.

‡ Measured on RDM-4 (H-2<sup>k</sup>) targets. Maximum <sup>51</sup>Cr released: 5,397 ± 140; spontaneous release: 773 ± 42 (14.3% of maximum). No cytotoxic activity was detected on EL4 targets; cytotoxic activity on RLβ-1 (H-2<sup>d</sup>) was significant only in the combination with 1 × 10<sup>6</sup> C anti-B6 stimulators, where maximal lysis was 12.7%.

to respond to C stimulator cells reflects the lack of an appropriate source of Th in that (a) peripheral T cells from all donors respond vigorously against C stimulators, and (b) thymocytes can respond when given an appropriate source of Th (not shown).

Because the restricting elements that control other known T cell interactions are encoded in the H-2 complex, it was of interest to determine whether T-T interaction measured in this system was also restricted by H-2 determinants. The B10 anti-C population was assayed for Th activity with thymocytes from mice expressing four independently derived H-2<sup>b</sup> haplotypes, which included 129, A.By, and C3H.SW, in addition to B6. The results are shown in Table VI. The helper population provided varying levels of help to thymocytes from all H-2<sup>b</sup> but not H-2<sup>k</sup> or H-2<sup>a</sup> donors. We have also found that these B10 anti-C Th cells do not interact with thymocytes from the B10 congenic partner B10.BR (H-2<sup>k</sup>) (not shown). Similar results have been obtained in other experiments and suggest that the interaction between Th cells and CTL.P is controlled, at least in part, by H-2-associated determinants. The differences between the degree of Th activity measured with C3H.SW thymocytes compared with B6 thymocytes (Table VI) is not considered significant because it did not occur in all other experiments (Fig. 2). However, the low levels of helper activity measured when using A.By thymocytes as a source of CTL.P have been a consistent observation. Whether this is an indication that the H-2<sup>b</sup> haplotype of A.By mice is a variant or that more than one determinant may control T-T interaction and A.By lacks one of these, is not known.



TABLE V  
*Syngeneic Preference of Helper T Cell-CTL Precursor Interaction*

Thymocyte donor (H-2 type)*	Number of long-term B10 anti- BALB/c cells added ( $\times 10^{-5}$ )‡	Percent specific $^{51}\text{Cr}$ released on targets§			
		P815		RDM-4	
		n	0.33 n	n	0.33 n
B6 (H-2 <sup>b</sup> )	0	6.4	3.6	—	—
B6 (H-2 <sup>b</sup> )	1	61.0	43.0	—	—
B6 (H-2 <sup>b</sup> )	3	62.5	44.9	—	—
B6 (H-2 <sup>b</sup> )	10	58.6	36.1	—	—
C3H (H-2 <sup>k</sup> )	0	0.0	0.9	—	—
C3H (H-2 <sup>k</sup> )	1	0.0	0.5	—	—
C3H (H-2 <sup>k</sup> )	3	0.0	0.8	—	—
C3H (H-2 <sup>k</sup> )	10	1.2	1.2	—	—
A (H-2 <sup>a</sup> )	0	0.0	1.0	—	—
A (H-2 <sup>a</sup> )	1	4.1	2.8	—	—
A (H-2 <sup>a</sup> )	3	3.8	1.1	—	—
A (H-2 <sup>a</sup> )	10	6.0	3.8	—	—
A.SW (H-2 <sup>a</sup> )	0	0.0	0.0	—	—
A.SW (H-2 <sup>a</sup> )	1	6.2	2.0	—	—
A.SW (H-2 <sup>a</sup> )	3	6.3	1.3	—	—
A.SW (H-2 <sup>a</sup> )	10	7.3	5.5	—	—
C (H-2 <sup>d</sup> )	0	—	—	0.0	0.0
C (H-2 <sup>d</sup> )	1	—	—	0.0	1.1
C (H-2 <sup>d</sup> )	3	—	—	16.7	11.8
C (H-2 <sup>d</sup> )	10	—	—	1.9	0.4

\* Thymocytes from all donors were cultured with  $\gamma$ -irradiated BALB/c spleen cells except those from BALB/c, which were stimulated with C3H.

‡ B10 anti-BALB/c cells were used as a source of Th cells 7 d after their seventh restimulation in vitro.

§ Cultures were washed and resuspended in 5 ml; 0.25 ml of these cells or of a 1:3 dilution were added to  $25 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells. Cytotoxicity was measured 5 d after initiation of culture. No cytotoxic activity was detected on EL4 (H-2<sup>b</sup>) targets in any of the cultures. Maximum counts per minute  $^{51}\text{Cr}$  released from P815:  $5,100 \pm 89$ ; spontaneous:  $1,180 \pm 43$  (23.1% of maximum). Maximum counts per minute  $^{51}\text{Cr}$  released from RDM-4:  $1,677 \pm 14$ ; spontaneous:  $422 \pm 26$  (25.2% of maximum).

|| — denotes not done.

*Is MHC-restricted T-T Interaction Only Observed with Cells Selected Through Multiple Restimulations?* Although the data presented in Tables V and VI demonstrate that T-T interaction in the generation of CTL may be H-2 restricted, it is possible that a restricted Th population is selected only after multiple restimulation and, in fact, may either not exist or represent only a minor component in unstimulated or once-primed populations. However, we have assayed once-primed Th populations with different thymocyte sources and have found that populations of helper cells with restricted activity can be observed after a single in vitro priming. In general, the restricted activity becomes more apparent with time after priming. In one such experiment, both the cytotoxic and Th activity of a once-primed B10 anti-C population (day 14 of culture) were measured in anti-C responses. Helper activity was assayed with thymocytes from B6, C3H.SW, C3H, A, and A.SW donors. As shown in Fig. 2, helper activity was expressed only when H-2<sup>b</sup> (B6 or C3H.SW) thymocytes

TABLE VI  
*Th Cell-CTL Precursor Interaction is Restricted by Self H-2-linked Determinants*

Thymocyte donor (H-2 type)*	Long-term B10 anti-C helper cells added‡	Percent specific <sup>51</sup> Cr released§		
		n	0.33 n	0.1 n
		%		
B6 (H-2 <sup>b</sup> )	—	13.3	5.2	0.0
B6 (H-2 <sup>b</sup> )	10 <sup>5</sup>	31.0	36.7	14.7
B6 (H-2 <sup>b</sup> )	10 <sup>6</sup>	41.1	25.1	8.6
129 (H-2 <sup>b</sup> )	—	9.3	1.2	0.1
129 (H-2 <sup>b</sup> )	10 <sup>6</sup>	27.6	15.0	4.7
A.By (H-2 <sup>b</sup> )	—	-3.1	-1.9	-1.8
A.By (H-2 <sup>b</sup> )	10 <sup>5</sup>	9.6	5.2	3.4
A.By (H-2 <sup>b</sup> )	10 <sup>6</sup>	2.7	-0.2	-0.2
C3H.SW (H-2 <sup>b</sup> )	—	-0.9	-3.6	-4.2
C3H.SW (H-2 <sup>b</sup> )	10 <sup>5</sup>	15.4	12.5	5.1
C3H.SW (H-2 <sup>b</sup> )	10 <sup>6</sup>	11.9	3.4	0.9
C3H (H-2 <sup>k</sup> )	—	-4.8	-2.8	-1.3
C3H (H-2 <sup>k</sup> )	10 <sup>5</sup>	-3.3	-4.1	-1.2
C3H (H-2 <sup>k</sup> )	10 <sup>6</sup>	-5.3	-3.3	-2.2
A.SW (H-2 <sup>a</sup> )	—	-6.1	-4.8	-2.3
A.SW (H-2 <sup>a</sup> )	10 <sup>5</sup>	-2.8	-4.2	-2.0
A.SW (H-2 <sup>a</sup> )	10 <sup>6</sup>	1.7	0.2	0.6

\* Thymocytes from all donors were cultured with  $\gamma$ -irradiated C stimulator cells.

‡ Used 7 d after their 10th restimulation in vitro.

§ Cytotoxic activity was assayed on P815 target cells. Maximum <sup>51</sup>Cr released: 4,523  $\pm$  71; spontaneous <sup>51</sup>Cr released: 1,109  $\pm$  22 (24.5% of maximum). Effector lymphocytes were titrated as detailed in Table V.

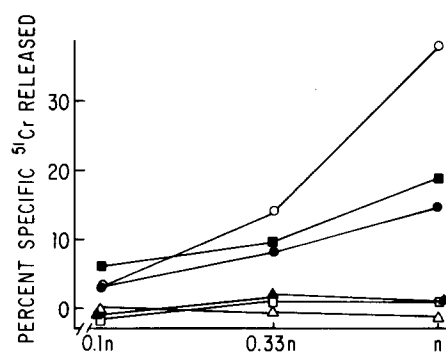


FIG. 2. Cytotoxic and helper activity of B10 anti-C cells 14 d after a single in vitro MLC. Cytotoxic activity was measured by restimulating  $10^6$  primed cells/ml against  $10^7$  C stimulators (○). Helper activity was measured by adding irradiated B10 anti-C cells to cultures of C stimulators and thymocytes from the following donors: (●) B6; (■) C3H.SW; (▲) A.SW; (□) C3H; (△) A. The results shown are with  $3 \times 10^6$  Th cells, but the helper population was titered over a 10-fold range without qualitatively affecting the results. Abscissa: dilution of culture tested for cytotoxic activity (Materials and Methods). Cytotoxicity was assayed against P815 target cells. Cytotoxic responses in the presence of Th cells are net cytotoxic activity above responses of thymocytes alone, if any. In general, cells from these cultures were negative. Maximum <sup>51</sup>Cr released from P815 targets: 9,372  $\pm$  116; spontaneous release: 1,194  $\pm$  17 (12.7% of maximum).

were used as a source of CTL.P. Note that the cytotoxic activity of the MLC-primed population is more pronounced than its helper activity.

### Discussion

The results presented demonstrate that there are at least two pathways by which Th cells can interact with CTL.P to give rise to active cytotoxic lymphocytes. The first of these is via an allogeneic effect that results from the recognition and response of Th cells to alloantigens on cells of the CTL.P-containing thymocyte population. This event is independent of the response of a Th cell to alloantigens on the stimulator cell (Tables III and IV). In this respect, the response is similar to that described for allogeneic interactions between T and B cells, in which Th cell activation is a consequence of recognition of alloantigens on B cells (12).

The second pathway is one in which T cell-T cell interaction is restricted by H-2 determinants (Table VI; Fig. 2) and, presumably, both CTL.P (18, 19) and Th cells (Table IV) (5) react against stimulator alloantigens. The demonstration of this type of interaction rested on our ability to identify primed Th populations with reduced ability to mediate allogeneic effects (Table V). That such a population can be identified leads us to two conclusions. First, not all cells that recognize alloantigens mediate allogeneic effects, and, therefore, the cell type responsible for this activity is a functional subpopulation of Th cells. This is not surprising because T cells that mediate allogeneic effects against B cells represent only a fraction of an alloreactive population as demonstrated by their restricted Lyt phenotype (20, 21) and limited frequency (12). Second, the fact that primed populations of Th cells can provide helper activity to syngeneic CTL.P in response to stimulator alloantigens, and yet may be inefficient in mounting an allogeneic effect against responder cells of the same H-2 type, suggests that these two helper activities are mediated, at least in part, by different Th cells.

It is not clear why primed populations tend to lose their capacity to mediate allogeneic effects on T cells. It is possible that the active subpopulation is unstable in culture or a new equilibrium is reached in which allogeneic effects are suppressed and/or not expressed under the conditions used. It is surprising that T cells in the stimulator population fail to mediate similar allogeneic effects, giving rise to CTL in thymocyte populations without addition of exogenous help. The failure to generate CTL against themselves suggests that T cells that mediate allogeneic effects exhibit some selectivity. This putative selectivity may be controlled at the cellular level or via the release of allogeneic effect factors with limited activity (22). It is also possible that such activity may exist, but that the culture conditions used favor negative allogeneic effects that suppress the allogeneic helper activity (12).

The quantity of Th cell activity measured in this system deserves comment because it is often quite low, particularly when unprimed helper cells are used. Pilarski (5) has shown that, for optimal activation of CTL with unprimed Th cells, as many as four- to eightfold more irradiated spleen helper cells must be added to thymocyte cultures. Our culture conditions do not allow us to use more helper cells than thymocytes because culture overcrowding occurs (Table II). With these conditions, we have found that the helper activity of unprimed allogeneic cells was consistently greater than that of unprimed syngeneic cells. This difference is, at least in part, a direct reflection of

the frequency of active Th cells as determined by limiting dilution analysis (K. A. Switzer and R. B. Corley. Manuscript in preparation.).

One parameter that can not be controlled in these studies is the effect of irradiation on Th cell function. It is possible that at least some of the unprimed helper cell precursors are radiosensitive (12). We have only been able to address the question of radiation sensitivity with the B10 anti-C helper line employed in some of the present studies (Tables V and VI). This line has lost its cytotoxic potential, which allows us to test its helper activity with and without irradiation. The level of cytotoxic activity generated from thymocytes is similar with either population, which suggests that primed Th cells are largely radioresistant (data not shown). These considerations suggest that, at least as a first approximation, the amount of Th activity and CTL potential measured in primed cultures (Fig. 2) reflect the number of active precursors mediating the respective functions, i.e., CTL are more frequent than Th cells. This has now been confirmed in limiting-dilution analysis; as in the thymus, Th cells are limiting in peripheral lymphoid organs (K. A. Switzer and R. B. Corley. Manuscript in preparation.). This fact has obvious implications for the multiplicity of CTL.P, which can be helped by a single Th cell.

Other studies in which restricted cytotoxic responses of lymphocytes from radiation chimeras to viral (23) or H-Y (24, 25) determinants have been investigated have led to the suggestion that Th cells are not involved in the generation of CTL to alloantigens. This argument was strengthened by the observations of von Boehmer and Haas (25) that fully allogeneic chimeras made CTL in response to foreign H-2 antigens. That Th cells for allospecific CTL responses exist and, moreover, can be restricted in their interaction with CTL.P clearly contrasts with predictions from the chimera models. It is possible that allospecific T cells, in contrast with viral or hapten-specific T cells, are not subject to the selective influences of the thymic epithelium. Unprimed T cell populations might include clones of Th cells capable of interacting with cells expressing various cell surface determinants. Th clones may, therefore, be restricted during selection and expansion after antigen presentation and/or as a result of the clones of CTL that respond. Alternatively, positive allogeneic effects may provide Th activity in alloimmunized chimeras.

It is not known whether the Th cells we are measuring act directly on cytotoxic precursors or by amplifying the activity of an intermediary cell. Thus we do not know at what level of interaction the MHC-restriction operates. We suggest, however, that the cellular events described here control the production of active Th cell-replacing factors that, upon release, may exert antigen nonspecific activity (6, 9, 10, 26, 27).

### Summary

The interaction of T helper (Th) cells with syngeneic and allogeneic cytotoxic T lymphocyte precursors (CTL.P) has been investigated. Unprimed and mixed lymphocyte culture-primed peripheral T cells were used as a source of Th. Thymocytes, which depend upon exogenous Th cells for activation, were used as a source of cytotoxic precursors. Data is presented that demonstrates that at least two pathways of T-T interaction can lead to the activation of cytotoxic lymphocytes. The first is an allogeneic effect, in which Th cells recognize and respond to alloantigens expressed on CTL.P. The second is the interaction of Th cells with syngeneic CTL.P, in which both cell types are thought to respond to alloantigens on stimulator cells. The latter

interaction can be shown to be restricted by H-2-linked determinants when primed Th cells are used and allogeneic effects against thymocytes are minimized. Restricted interactions between unprimed Th cells and thymocyte CTL.P have never been observed. Mechanisms that may explain the difference between the interaction of unprimed and primed Th cells with CTL.P are discussed.

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