

A REQUIREMENT FOR ANTIGEN-SPECIFIC HELPER T CELLS IN THE GENERATION OF CYTOTOXIC T CELLS FROM THYMOCYTE PRECURSORS*

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Although cooperative interactions between helper T cells and B-cell precursors have been extensively characterized (1, 2), the role of helper T cells in the triggering and differentiation of cytotoxic T-cell precursors remains ambiguous. Several laboratories have described synergistic interactions between T cells recognizing H-2K or D antigens (SD) and T cells recognizing Ia antigens (LD); increased anti-H-2-directed cytotoxicity is generated if both H-2K or D and Ia antigens are foreign to the responding T cell (3-5). This suggests that cooperation between anti-Ia helpers and anti-H-2 killer precursors augments cytotoxicity. However, this type of cooperation must not be essential since Forman and Klein, and Klein et al., have shown that cytotoxicity is generated when the only antigenic difference between responder T cells and stimulator cells is derived from a mutation in the *H-2D* or *H-2K* end of the histocompatibility gene complex (i.e., there is no detectable Ia antigenic difference between the stimulator and responder cells) (6, 7).

A different type of cooperative interaction in the induction of cytotoxic T-cell precursors has been observed by both Cohen and Howe (8) and by Wagner (9). In these experiments, lymph node responder cells were co-cultured with syngeneic thymocytes as well as allogeneic stimulator cells. A cytotoxic response was obtained from low doses of lymph node cells co-cultured with thymocytes and stimulator cells, although neither cell population responded well alone. Unfortunately, these experiments did not allow a clear-cut definition of the helper effect since the cytotoxic cells produced were derived from both lymph node and thymocyte populations (9) and the antigen specificity of the interaction was not investigated. More recently, Plate has described a soluble factor which augments the cytotoxicity generated by a helper T-cell-depleted culture, but again the specificity of the interaction was not investigated and the helper factor was not an essential requirement for the generation of cytotoxicity (10).

In this paper we describe an *in vitro* system in which helper T cells are an absolute requirement for the generation of cytotoxicity. Thymocyte killer precursors are not stimulated to yield cytotoxic cells unless irradiated splenic helper cells are added to the cultures. These helper cells are antigen-specific, radio-resistant, T cells. However, when the strain specificity of these helpers was analyzed, they were found to be able to cooperate across allogeneic barriers. This is similar to the lack of restriction displayed by unprimed T cells cooperating *in vitro* (11), in tetra parental mice (12), and cells that were primed in a chimeric environment (13). Since stringent strain specificity of help seems to be important mainly when T cells are primed before contact with the allogeneic precursor cell (14, 15), the finding that helper T cells from normal spleen are not

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strain specific is consistent with the general pattern observed for cooperation in humoral responses. In further experiments, it is shown that a hapten-carrier relationship within the antigen is as important for T-helper-T-killer interactions as it is for T-B interactions.

Materials and Methods

Animals. Female and male mice aged 5–14 wk of the following strains were obtained from the University of Alberta animal breeding facility: CBA/CaJ, BALB/cCr, C3H-NBSn, C3H-SWSn, C3H-HeJ; C57BL/6J, (BALB/c × C57BL/6)F₁, DBA/1J, and AKR/J were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Cell Lines P815 mastocytoma (*H-2^d*, derived from DBA/2) and E14 leukemia (*H-2^b*, derived from C57BL/6) were a gift from Dr. E Sabbadini, University of Manitoba, Canada, and were maintained in vitro.

Materials F15 (Eagle's minimal essential), H16 (Dulbecco's modified), and Leibovitz media were obtained in powder form from Grand Island Biological Co., Grand Island, N. Y., who also supplied the fetal calf serum (FCS).¹ Gentamicin was from Microbiological Associates, Bethesda, Md. Irradiation was via a ¹³⁷Cs source (gamma cell 40, Atomic Energy of Canada Ltd): dose rate = 100 rads per min

Tolerant Mice CBA/CaJ cells tolerant of BALB/c antigens were prepared by reconstitution of 1,000 rad irradiated BALB/c mice with 2×10^7 CBA/CaJ bone marrow cells (13). Spleens were removed from these mice 2–3 mo later for use as helper cells. These spleen cells were 100% CBA cells as measured by cytotoxicity of anti-H-2^k antiserum plus complement. The CBA cells derived from these chimeric mice were tolerant in that they were unable to generate an anti-BALB cytotoxic response, although they did yield normal anti-C57BL/6 cytotoxicity

Culture Technique. The method for culturing mixed lymphocytes has been described previously² and utilized Marbrook acrylamide tissue culture vessels (rafts) (16). These rafts "float" in a 15 × 60-mm Petri dish containing 4 ml of medium + FCS. The inner chamber of the raft is subdivided into 36 individual wells and holds a total vol of 1 ml. Although each well contains a cell pellet, the supernatant medium is contiguous for all wells in a raft.

Rafts were prepared as described by Marbrook and Haskill (16). They were washed with four overnight changes of saline, autoclaved, and then stored at 4°C. 2 days before their use in culture, rafts were equilibrated with two overnight changes of medium (F15 without FCS).

Cell Preparation and Culture Responder thymus cells (from 4–5 wk CBA/CaJ mice), splenic stimulator cells (from 8–14 wk BALB/c mice), and splenic helper cells (from 12–14 wk CBA/CaJ mice unless otherwise indicated) were removed aseptically and minced in Leibovitz medium + 10^{-4} M mercaptoethanol (ME) + 10% FCS. Both stimulator and helper spleen cell populations received identical treatment consisting of a 90-min preincubation at 37°C followed by 1,000 rads of irradiation as described by Lafferty et al. (17). Immediately before culture, cells were washed and resuspended in F15 plus 10^{-4} M ME, 10% FCS, and 50 µg/ml gentamicin. Thymocyte responder cells ($0.5\text{--}4 \times 10^6$) were mixed with the indicated number of irradiated stimulator cells ($4\text{--}16 \times 10^6$), plus or minus splenic helper cells ($0.5\text{--}8 \times 10^6$), made up to a vol of 1.0 ml and pipetted into a raft. Cultures were incubated at 37°C in 10% CO₂-air atmosphere for 5 days. The time-course of a thymocyte response to alloantigens was found to parallel that of a lymph node response with maximal cytotoxicity generated on day 5 of culture. Each experiment reported in this work was repeated a minimum of three times, except for the experiment reported in Fig. 2 which was repeated twice.

Cytotoxicity Assay Cells from the rafts were harvested, centrifuged, and resuspended in 1 ml of fresh F15 + ME + FCS. The cytotoxicity assay was performed in V-bottom microtiter trays. Each assay consisted of 0.1 ml of ⁵¹Cr-labeled target cells (10^5 cells) plus 0.1 ml of medium or cell suspension. Trays were incubated at 37°C in 10% CO₂-air for 4 h. The top 0.1 ml was removed and counted for supernate ⁵¹Cr cpm. All assays were set up in triplicate at each dilution of cultured

¹ Abbreviation used in this paper FCS, fetal calf serum.

² Pilarski, L M, and L V Borshevsky 1977. A new system for highly efficient generation of alloantigen-specific cytotoxic T cells. *Eur J. Immunol.* In press.

lymphocytes. Total release of ^{51}Cr was determined by incubation of 0.1 ml of 0.05% Triton X-100 detergent with 0.1 ml (10^5) ^{51}Cr targets. Spontaneous lysis was determined by incubating 0.1 ml of targets with 0.1 ml of medium. In both cases the top 0.1 ml was removed after 4 h at 37°C and counted in a gamma counter.

To accurately determine the cytotoxicity of a population of cells, several dilutions of those cells were always assayed. In general the cultures were assayed at $1/10$, $1/30$, and $1/60$ of a culture. This range insured that at least two of the dilutions would fall in the linear portion of the curve derived from plotting the percent specific ^{51}Cr release versus the dose of killer lymphocytes, where the amount of killing is directly proportional to the number of lymphocytes added. Results are generally expressed as follows:

$$\% \text{ specific release} = \frac{\text{sample cpm} - \text{spontaneous lysis cpm}}{\text{detergent lysis cpm}} \times 100.$$

Calculations of the cytotoxic efficiency used the number of cells originally cultured rather than the number of viable cells recovered after 5 days of culture. This was because (a) the cytotoxic levels generally correlated better with the original number rather than the viable number and (b) because use of viable cells recovered effectively "rewards" cultures with the most cell death. The number of cells originally cultured is also used to calculate lymphocyte to target ratios which range from 0.16:1 to 4:1. Actual detergent and spontaneous release values are included in the table or figures for each experiment reported.

Culture and Preparation of Target Cells. P815 mastocytoma was maintained in H16 + 10% FCS + 75 $\mu\text{g/ml}$ gentamicin. EL4 leukemia was maintained in F15 + 10^{-4} M ME + 10% FCS + 75 $\mu\text{g/ml}$ gentamicin. 2 days before use, cultures were set up at $5 \times 10^4/\text{ml}$ for P815 and $1 \times 10^5/\text{ml}$ for EL4. When harvested 2 days later, both cultures had concentrations of $3-8 \times 10^5/\text{ml}$ which was found to be optimal for low spontaneous release volume.

The desired number of tumor cells were then centrifuged and resuspended in medium (-FCS) at a concentration of 5×10^6 cells/ml. Radioactive $\text{Na}^{51}\text{CrO}_4$ was added to a final concentration of 100 $\mu\text{Ci/ml}$ and the cells allowed to incorporate label for 60 min at 37°C . They were then washed four times in F15 + ME + FCS and resuspended at a concentration of $1 \times 10^6/\text{ml}$. These cells were then added to the cytotoxic lymphocytes at 0.1 ml/well (10^5 targets).

Anti-Theta Serum Preparation and Treatment. AKR anti-CBA theta serum was prepared by the method of I. Ramshaw (personal communication). This involved injection of AKR mice with 5×10^7 CBA thymocytes intravenously. The mice were then bled on day 7 after injection to yield a preparation of anti-theta serum which was effective in killing $1-2 \times 10^7$ lymph node T cells at a final dilution of $1/6$, and killed thymocytes at a dilution of $1/50$ (as measured by dye exclusion). It had no effect on antibody-forming cells or B cells. The treatment was as follows: lymphocytes harvested from rafts were resuspended in medium containing anti-theta serum or normal serum at $1/6$ dilution and allowed to stand at room temperature for 20 min. Then rabbit complement preabsorbed with mouse tissues was added to a final dilution of $1/15$ and the mixture incubated at 37°C for 45 min. This procedure yielded efficient killing of cytotoxic T cells with essentially no nonspecific killing (see Fig. 2, untreated cells compared to cells treated with normal mouse serum plus complement).

Results

Conditions for Generation of Cytotoxic Cells from Thymocyte Precursors. CBA thymocytes co-cultured with irradiated BALB/c spleen cells do not yield an anti-H-2D cytotoxic response (Fig. 1). However, when irradiated CBA spleen cells were added to CBA thymocyte responder cells plus BALB/c stimulator cells, cytotoxic cells were generated after 5 days of culture. Fig. 1 illustrates a typical experiment in which CBA thymocytes were cultured with BALB/c stimulator cells and with or without irradiated syngeneic spleen cells. To compare different groups, a cell dose-response of percent lysis versus the number of lymphocytes was plotted. In the absence of irradiated syngeneic spleen cells, the progeny of 10×10^4 thymocytes yield no cytotoxicity; in

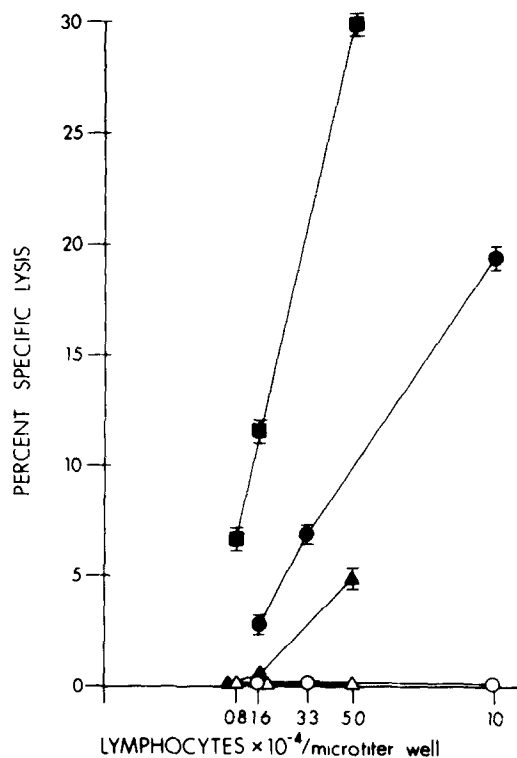


FIG 1 Production of cytotoxic T cells as a function of thymocyte responder cell dose and the presence of helper activity. In this figure the degree of target cell lysis is plotted as a function of the number lymphocytes included in the assay. The indicated number of CBA thymus cells, or lymph node cells, were incubated with irradiated BALB/c splenic stimulator cells, plus or minus irradiated CBA spleen cells, for 5 days and the progeny of these responder cells assessed for cytotoxic activity. Cytotoxicity was detected by measuring release of ^{51}Cr from 10^5 -labeled P815 target cells. (○—○) 1×10^6 CBA thymus cells plus 16×10^6 irradiated BALB/c spleen cells. Viable cells = 1×10^5 /culture (△—△) 5×10^5 CBA thymus cells plus 16×10^6 irradiated BALB/c spleen cells. Viable cells = 2.4×10^5 /culture. (■—■) 5×10^5 CBA lymph node cells plus 8×10^6 irradiated BALB/c spleen cells. Viable cells = 6.4×10^5 /culture. (●—●) 1×10^6 CBA thymus cells plus 8×10^6 irradiated BALB/c spleen cells, plus 8×10^6 irradiated CBA spleen cells. Viable cells = 3.8×10^5 /culture (▲—▲) 5×10^5 CBA thymus cells plus 8×10^6 irradiated BALB/c spleen cells, plus 8×10^6 irradiated CBA spleen cells. Viable cells = 1.6×10^5 /culture. Points represent the mean \pm standard deviation. Detergent lysis = $6,863 \pm 143$. Spontaneous lysis = 765 ± 17 . Machine background of 142 ± 11 has not been subtracted.

contrast the progeny of only 5×10^4 lymph node responders caused 30% specific lysis of the target cells. In the presence of irradiated syngeneic spleen cells a thymocyte cytotoxic response occurs yielding 5–17% specific lysis. The cytotoxic response generated from thymocyte precursors was proportional to the number of thymocytes originally cultured. A comparison between the response of comparable numbers of thymocytes or lymph node cells showed that cultures of lymph node cells were approximately sixfold more cytotoxic than were cultures of thymocytes.

Cytotoxicity in Cultures Containing Irradiated syngeneic spleen Cells is Derived from Thymocytes. Cytotoxic Cells Bear the Theta Antigen. Although

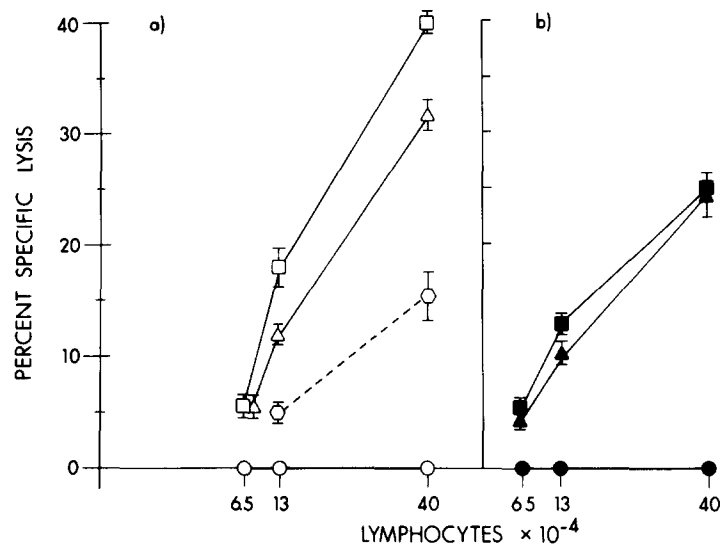


FIG. 2. Cytotoxic activity is derived from the thymocyte responder cell. 4×10^6 CBA thymus cells were cultured with 8×10^6 irradiated BALB/c spleen stimulator cells. In addition cultures received either (a) 8×10^6 irradiated CBA spleen cells or (b) 8×10^6 irradiated AKR spleen cells. On day 5, cultures were harvested, divided into three aliquots, and left untreated, treated with normal serum plus complement, or treated with AKR anti-CBA theta serum plus complement (2a) CBA thymus cells plus irradiated CBA helper spleen cells. (\square — \square) Untreated: viability = 10.4×10^5 cells/culture. (Δ — Δ) Treated with normal serum plus complement: viability after treatment = 10×10^5 cells/culture. (\circ — \circ) Treated with AKR anti-CBA theta serum plus complement: viability after treatment = 0.4×10^5 cells/culture. (\square — \square) Thymus cells + 16×10^6 irradiated BALB/c spleen cells (no additional helpers were present). Viable cells = 6.2×10^5 /culture (untreated) (2b) CBA thymus cells plus irradiated AKR helper spleen cells. (\blacksquare — \blacksquare) Untreated: viability = 9.2×10^5 cells/culture. (\blacktriangle — \blacktriangle) Treated with normal serum plus complement: viability after treatment = 5×10^5 cells/culture. (\bullet — \bullet) Treated with anti-theta serum plus complement: viability after treatment = 0.2×10^5 cells/culture. This experiment was repeated in full using 1×10^6 thymus cells as responder cells, and the same pattern was observed with two major differences: (a) cultures containing the lower dose of responders were less cytotoxic (maximum = 14% specific lysis at 1:1 lymphocyte to target ratio) and (b) cultures of 10^6 thymocytes were not cytotoxic in the absence of added helper spleen cells. However, all the cytotoxicity was removed by treatment with AKR anti-CBA theta serum plus complement, and unaffected by treatment with normal serum plus complement regardless of whether helper cells were AKR or CBA spleen cells. Points represent the mean \pm standard deviation of counts per minute released from 10^5 ^{51}Cr -P815. Detergent lysis = $9,611 \pm 768$. Spontaneous lysis = 768 ± 13 . Machine background of 123 ± 7 has not been subtracted.

control cultures containing only mixtures of irradiated CBA and BALB spleen were always negative, the presence of large numbers of syngeneic irradiated spleen cells (8×10^6) made it necessary to experimentally show that the cytotoxicity measured was derived from thymocyte precursors and not from a thymocyte-stimulated nonproliferative differentiation of the irradiated CBA spleen cells responding to the irradiated BALB/c stimulators. To demonstrate the source of our cytotoxicity, thymocytes were cultured with BALB/c stimulators and either irradiated CBA spleen cells (Fig. 2a) or irradiated AKR spleen cells (Fig. 2b); AKR cells share H-2 antigens with CBA but bear a different θ -antigen (18). The resulting cultures were then assayed for (a) augmented

cytotoxicity in the presence of CBA or AKR spleen cells and (b) the presence of CBA theta antigen on the cytotoxic effector cells. In this experiment there was some indigenous helper activity in the cultures containing 4×10^6 thymocytes (5% lysis by the progeny of 13×10^4 lymphocytes), which was considerably increased by addition of CBA spleen cells (18% lysis by the progeny of 13×10^4 lymphocytes) (Fig. 2a). This cytotoxicity was abolished by treatment with anti-theta serum plus complement, but was unaffected by treatment with normal serum and complement. This establishes that the cytotoxicity was due to a theta-bearing cell. Similarly Fig. 2b shows that irradiated AKR spleen cells were also able to help the cytotoxic response although to a lesser degree than CBA cells. In this case as well, the cytotoxicity was completely abolished by AKR anti-CBA theta serum, indicating that all of the killer cells were derived from the CBA thymocytes and not from the irradiated spleen cells. Likewise, when 1×10^6 thymus cells were co-cultured with CBA or AKR irradiated spleen cells and BALB/c stimulators, all of the activity observed was sensitive to treatment with anti-theta serum and was derived from the CBA thymocyte responder cells.

Helper Activity is Due to a Theta-Bearing Spleen Cell. To establish that the augmentation of thymocyte cytotoxic responses by syngeneic spleen cells was a true helper effect, it was necessary to show that it was T-cell dependent. Fig. 3 shows that the helper activity was completely removed by treatment with anti-theta serum and complement, but was unaffected by treatment with normal serum plus complement. This indicates that the activity is T-cell dependent.

Number of Helper Cells Required for the Generation of Maximum Cytotoxicity. Several doses of irradiated helper cells were tested for their ability to help a thymocyte cytotoxic response (Table I). Optimal help was present in populations of $4-8 \times 10^6$ irradiated normal spleen cells; 1×10^6 cells contained very few helper cells. The number of BALB/c stimulator cells required for a maximum response was also tested since previous work had shown that 16×10^6 irradiated spleen cells were optimal to stimulate lymph node-derived precursors. Lines three and seven of Table I compare the thymocyte cytotoxic response in the presence of helper cells, when 8 or 16×10^6 stimulator cells were present. Identical cytotoxic responses were observed at both numbers which indicated that a broad range of stimulator cell doses were effective when cultures contained helper cells.

The Splenic Helper T Cell is Antigen-Specific: Spleen Cells Tolerant of BALB/c Antigens are Deficient in Anti-BALB/c Helper Activity. Inasmuch as the helper T cells described here are resident in populations of normal spleen cells, it was expected that the help would be multi-clonal; that is normal spleen cells should contain helpers that help an anti-C57BL/6 response as well as helpers for an anti-BALB/c response. When CBA thymocytes were cultured with either BALB/c stimulator cells or C57BL/6 stimulator cells, with irradiated normal CBA spleen cells as helpers, it was found that irradiated normal spleen provides help for both anti-BALB and anti-C57BL/6 responses with the optimal dose of helper cells for both responses being $4-8 \times 10^6$ normal spleen cells (data not shown, and see Fig. 4b).

If the helper activity is specific, a testable prediction can be made that CBA

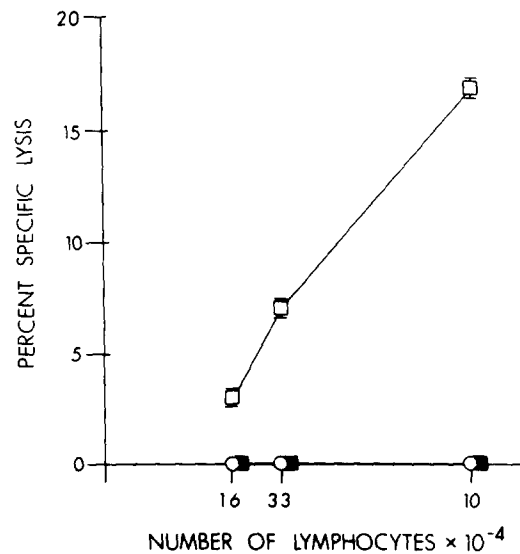


FIG. 3. Helper activity is dependent on a theta antigen-bearing cell. 5×10^5 CBA thymocytes were cultured with 4×10^6 irradiated BALB/c stimulator cells plus normal serum plus complement-treated or anti-theta serum plus complement-treated irradiated CBA spleen cells (12×10^6 /culture). Normal CBA spleen cells had 50% survival after anti-theta treatment; 100% of cells survived the normal serum treatment. The number of CBA spleen cells added to cultures was calculated based on the number of viable cells present before treatment. (□-□) Thymus cells plus normal serum-treated CBA helper cells. (■-■) Thymus cells plus anti-theta serum-treated CBA helper cells. (○-○) Thymus cells with no CBA helper cells. Points represent the mean \pm standard deviation of counts per minute released from 10^5 ^{51}Cr -labeled P815 target cells. Detergent lysis = $4,002 \pm 118$. Spontaneous lysis = 600 ± 42 . Machine background of 185 ± 8 has not been subtracted.

TABLE I
Optimal Conditions for Maximum Generation of Cytotoxicity

Group	Irradiated spleen cells $\times 10^{-6}$		Percent specific ^{51}Cr cpm released by lymphocytes $\times 10^{-4}$		
	BALB/c stimulator cells	CBA helper cells	16	3.3	10
1	16	—	0	0	1.0
2	8	12	3.9	9.3	17
3	8	8	4.2	9.8	24.5
4	12	4	6.7	12.3	28.2
5	15	1	0	1.0	3.8
6	16	0.5	0	1.5	2.9
7	16	8	5.3	12.5	25.5
8*	16	8	0	0	0

1×10^6 CBA thymocytes were cultured with the indicated number of irradiated stimulator and helper spleen cells for 5 days. The anti-BALB/c response was measured by the amount of label released from 10^5 ^{51}Cr -labeled P815 target cells. Viable cells at harvest ranged from $3-6 \times 10^5$ per culture. Detergent lysis = $2,729 \pm 93$; spontaneous lysis = 257 ± 13 . Machine background of 71 ± 7 has not been subtracted.

* Irradiated cell control, no CBA thymocytes were added to this group.

spleen cells which are tolerant of $H-2^d$ antigens should be deficient in anti-BALB/c helper cells, but should contain normal numbers of anti-C57BL/6 helper cells. This prediction was experimentally verified in the following way.

CBA spleen cells tolerant of BALB/c antigens were prepared by reconstituting irradiated BALB/c mice with CBA bone marrow cells. Spleen cells were collected 2 mo later and characterized for $H-2$ markers as well as the specificity and degree of tolerance as described in Materials and Methods. These cells were then assessed for anti-BALB/c and anti-C57BL/6 helper activity. Fig. 4 illustrates the depletion of anti-BALB/c helpers, and the presence of normal numbers of anti-C57 helpers in tolerant spleen cells. CBA thymocytes were cultured with irradiated BALB/c stimulator cells plus either normal or tolerant irradiated helper spleen cells (Fig. 4a). The progeny of 3.3×10^4 thymocytes which had been co-cultured with irradiated spleen cells yielded 31.5% specific lysis of P815 targets. In contrast, thymus responder cells co-cultured with tolerant spleen cells equivalent in number to the normal cells yielded essentially no anti-P815 cytotoxicity by 3.3×10^4 cells and only marginal lysis (4.2%) by 1×10^5 cells. Thus normal spleen cells helped a response at least 30-fold more effectively than did tolerant spleen cells.

I then asked if the defect in tolerant spleen was specific. A (BALB \times C57) F_1 cell was chosen as a stimulator cell (antigen) for these specificity controls because it afforded several advantages over use of a stimulator cell which was completely allogeneic to the BALB/c stimulator cell. Firstly, it provided a normal specificity control to determine if cells tolerant of BALB/c antigens contained anti-C57 helpers capable of helping an anti-C57 killer cell response. Unlike a completely allogeneic stimulator cell, however, use of the (BALB \times C57) F_1 cells would provide an internal control for any anti-BALB/c inhibitory activities which could have been present in the tolerant cells (see Discussion). Equally important was the fact that both an anti-BALB/c and an anti-C57 response could be measured; this controls for such problems as a possibly different frequency (or different induction requirements) of anti-C57 killer precursors as compared to anti-BALB precursors in the thymocytes. This is important since in general the anti-C57 response of thymocytes was lower than an anti-BALB response when the appropriate stimulatory and helper cells were used. The stimulation of an anti-BALB response when using an F_1 cell as antigen would also give indications of the mechanism of help.

Thymus responder cells were cultured with (BALB/c \times C57BL/6) F_1 irradiated stimulator cells and either normal spleen cells or spleen cells tolerant to BALB/c antigens (Fig. 4b). Both the anti-BALB/c ($H-2^d$) and the anti-C57BL/6 ($H-2^b$) responses were measured. The anti-C57BL/6 response was essentially the same whether normal spleen or tolerant spleen was used as the source of helpers (8.9% lysis; Fig. 4b, triangles). The anti-BALB response also was similar for both normal spleen (26% lysis) or tolerant spleen (22% lysis). This indicates that irradiated tolerant spleen could only help an anti-BALB response when both BALB antigens and C57BL/6 antigens were present on the stimulator cell (Fig. 4b), not when BALB/c determinants were the only antigens available for the helper cells to recognize (Fig. 4a).

Strain Specificity of Helper Cells. Inasmuch as B-cell-T-cell cooperation

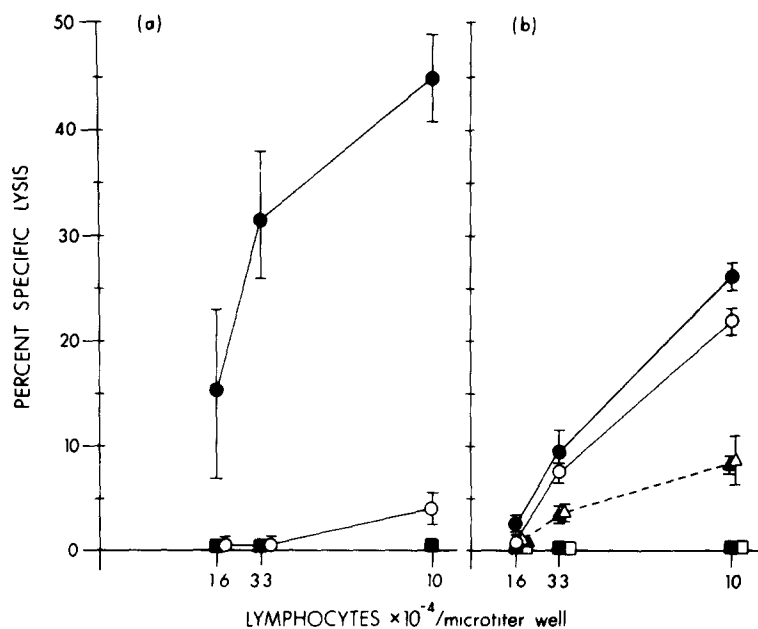


FIG. 4. Absence of specific anti-BALB/c helper activity in CBA spleen cells tolerant of BALB/c antigens. CBA thymus cells (1×10^6 cells/culture) were co-cultured with irradiated stimulator cells (8×10^6 cells if helper cells were added, 16×10^6 cells if no extra cells were added) and either normal or tolerant CBA spleen cells (see Materials and Methods) at 8×10^6 cells/culture. Harvest was at day 5. Part (a): Thymus cells plus BALB/c irradiated stimulator cells. Cultures were assayed on P815 target cells (cross-reactivity of the response was measured on EL4 target cells, but since there was minimal cross-reaction here, the data is not presented). (■—■) Thymus cells with no irradiated CBA spleen cells added. (●—●) Thymus cells plus 8×10^6 irradiated normal CBA spleen cells. (○—○) Thymus cells plus 8×10^6 irradiated tolerant CBA spleen cells. Part (b): Thymus cells plus (BALB/c \times C57BL/6)F₁ irradiated stimulator cells. Cultures were assayed on P815 target cells to measure the anti-BALB/c response and on EL4 to measure the anti-C57BL/6 response. (■—■) Anti-BALB response, and (□—□) anti-C57BL response by thymus cells with no irradiated CBA spleen cells added. (●—●) Anti-BALB response, and (▲—▲) anti-C57BL response by thymus cells plus 8×10^6 irradiated normal CBA spleen cells. (○—○) Anti-BALB response, and (△—△) anti C57BL response by thymus cells plus 8×10^6 irradiated tolerant CBA spleen cells.

demands stringent histocompatibility matching for an efficient response for primed cells (14, 15), except in special circumstances (13), but not for unprimed cells (11, 12), we were interested in analyzing the histocompatibility requirements for T-helper cell-T-killer cell collaboration. If helpers for killers were *H-2* gene complex-restricted in their ability to cooperate, then we would predict the mixtures of *H-2^a* thymocyte precursors (CBA) with histoincompatible helper spleen cells (*H-2^q*, *H-2^b*, *H-2^v*, and *H-2^d*) and stimulator cells would not yield cytotoxicity. A positive result, the generation of cytotoxicity, would indicate a lack of restriction on the part of the helper T cells. These predictions were tested in the experiment of Fig. 5 where we found that the helper T cells which collaborate with cytotoxic T-cell precursors (*H-2^a*) were not strain specific. Several doses of each type of allogeneic helper cell were tested, but since the

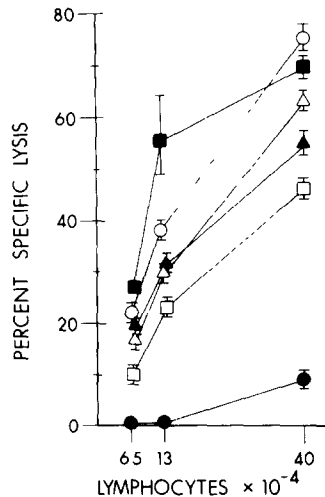


FIG. 5. Strain specificity of helper T cells. 4×10^6 CBA thymocytes ($H-2^k$) were cultured with 8×10^6 BALB/c stimulator spleen cells plus irradiated helper spleen cells of various $H-2$ haplotypes. Assay was at day 5 on ^{51}Cr -labeled P815 target cells. (●—●) Plus 8×10^6 BALB/c spleen cells ($H-2^d$). (○—○) Plus 8×10^6 CBA spleen cells ($H-2^k$). (△—△) Plus 8×10^6 C3H-NBSn spleen cells ($H-2^p$). (▲—▲) Plus 8×10^6 C3H/SwSn spleen cells ($H-2^b$). (■—■) Plus 4×10^6 DBA/IJ spleen cells ($H-2^q$). (□—□) Plus 8×10^6 DBA/IJ spleen cells ($H-2^r$). Detergent lysis = $4,276 \pm 102$ cpm spontaneous lysis = 375 ± 40 cpm.

helper cell dose-response pattern was essentially the same for all helper haplotypes, only the response of thymocytes ($H-2^k$) collaborating with 8×10^6 helpers is presented. It is interesting that helper cells bearing $H-2^k$, $H-2^p$, and $H-2^b$ antigens were equally efficient in their ability to collaborate with $H-2^k$ killer precursors. $H-2^q$ -bearing helper cells, however, seemed reproducibly more efficient in that fewer cells were required for a maximum response. The $H-2^q$ helper cells did have a different background genetic constitution than the other allogeneic helpers, but this was unlikely to be an important factor since cells from the C57BL/10 mouse ($H-2^b$), which also has a different background, yielded the same levels of help as did the C3H congenic $H-2^b$ cell populations (data not shown). A relevant observation in terms of the mechanism by which allogeneic cells help was the fact that, in general, BALB/c ($H-2^d$) spleen cells were completely unable to help an $H-2^k$ anti- $H-2^d$ cytotoxic response (Figs. 1 and 3) although occasionally a low response was obtained at high thymocyte doses (Figs. 2, 4, and 5). This was a reproducible finding in a large number of experiments. The fact that the degree of cytotoxicity observed in the presence of only irradiated BALB/c spleen cells depends exclusively on the thymocyte dose suggests that the helper cells are derived from the CBA thymocytes rather than from the BALB/c spleen cell population. Thus an $H-2$ incompatible cell which is tolerant of the stimulator cell antigens was unable to cooperate with the killer precursor (i.e., BALB/c helper cells cannot recognize BALB/c antigens).

Discussion

This work documents the requirement for antigen-specific helper T cells in the generation of a cytotoxic T-cell response. Although others have reported the

involvement of collaborating T cells in cytotoxic T-cell responses, they have not been able to show that these cells are essential for a response (3-10). The ability to do so here was dependent on three factors: (a) the development of a new method for the generation of cytotoxicity, (b) the use of normal thymocytes as a source of precursor cells relatively uncontaminated with active helper cells, and (c) the discovery that irradiated normal spleen cell populations contained sufficient functional helper cells to induce a cytotoxic response by the thymocyte precursors. The helper T cells described here have some of the same functional properties as the T cells which help B cells. That is, they are antigen-specific, theta-bearing, radio-resistant cells resident in spleen. They are also similar to helpers for B cells in an unprimed situation (13, 15) and some primed helpers (14) in that they do not display strain specificity. However, the helper cells are sufficiently numerous and sufficiently differentiated to be detected in irradiated normal spleen. In general, priming of helper function is necessary to expand clones and produce radio-resistant functional cells if one measures their function in a B-cell assay (19-21). If the same type of helper cell participates in both B- and T-cell responses, as seems probable based on surface markers (both are $ly\ 1^+$ (3, 22)), then it appears that normal spleen contains numbers of helpers adequate to collaborate with alloantigen-reactive cytotoxic T-cell precursors but not sufficient for induction of xenoantigen-reactive B-cell precursors. Two obvious interpretations of this finding can be advanced. Firstly, since there appears to be a very high frequency of some cells capable of responding to alloantigens (Simonsen phenomenon, 23, 24), it is possible that the frequency of helper T cells specific for alloantigens is also unusually high. The strongest argument against this view is that no helper cells were detectable in 1×10^6 spleen cells as seen in Table I. This low frequency is in apparent contrast to the results of Skinner and Marbrook (24) who found that the frequency of alloantigen responsive units was 1 in 1,700, in close agreement with an estimate of 1 in 2,100 found in our system (Pilariski, manuscript in preparation). The helper frequency seen here seems more compatible with the number of cells capable of responding to xenoantigens than with the frequency of alloantigen-responsive units. However, this is at best an imprecise estimate of helper frequency since the sensitivity of detection of helper cells in this system is not known. The second interpretation is that the induction of cytotoxic T-cell precursors requires fewer helper T cells than does the induction of a B-cell precursor. Bretscher's immune class regulation theory predicts that precursors of cell-mediated immunity will require less signal (2) for induction than will precursors of humoral immunity. (25). More experimental work is required to differentiate between these two alternative explanations for the presence in normal spleen of radio-resistant helper T cells able to collaborate with killer cell precursors.

Since the conclusions drawn here are fundamental to an understanding of the mechanisms underlying immune induction, it was important to firmly establish that the cytotoxicity observed in the presence of irradiated helper cells was derived from the thymocyte population and not from the irradiated spleen cells. Although the control groups containing only irradiated cell mixtures were always negative for cytotoxic activity and it seemed highly unlikely that irradiated cells could be stimulated into a nonproliferative differentiation to cytotoxic effector cells, further confirmation was sought. In Fig. 2 we demonstrated that

the cytotoxic effector cells derived from a culture containing CBA thymocytes, AKR-irradiated helper cells, and irradiated BALB/c stimulator cells uniformly bore the theta allele of CBA. This indicates that all effector cells derived from the CBA thymocytes and not from the irradiated AKR helper cell population which bears a different theta antigen (18).

Also of fundamental importance is the demonstration that the helper cells were antigen specific. Because the helpers were resident in irradiated normal spleen, it was expected that they might misleadingly appear to be nonspecific when in fact the unprimed splenic helper population was probably multiclonal at least in terms of alloantigen-specific cells. This expectation was borne out by the observation that irradiated spleen contained both anti-BALB/c and anti-C57BL/6 helpers at approximately equal frequency as determined by function. Therefore, if help was specific, a spleen cell population tolerant of BALB/c antigens should be deficient in anti-BALB/c helpers, but possess normal numbers of anti-C57BL helper T cells. Spleen cells from irradiated BALB/c mice reconstituted with CBA bone marrow cells 2 mo before use were the source of CBA helper cells tolerant of BALB/c antigens. These cells were unable to help a CBA thymocyte response to BALB/c stimulator cells, but, in contrast, were able to efficiently help an anti-BALB/c response to (BALB/c \times C57BL)F₁ stimulator cells which possess C57 determinants for helper recognition. An interpretation of this is diagrammatically presented in Fig. 6 where precursor cells are shown recognizing BALB/c determinants in collaboration with helper cells recognizing C57BL determinants on the stimulator cell. Thus, as in T-cell-B-cell cooperation, the BALB/c antigenic determinants function as the haptenic groups, and the C57BL determinants function as the carrier moieties. This is consistent with a requirement for associative recognition of linked determinants in the induction of cytotoxic T-cell precursors and therefore comparable to the recognition events occurring in other forms of cooperation.

Inasmuch as the tolerant helper cells were derived from bone marrow radiation chimeras it was necessary to characterize the spleen cells of these animals for (a) H-2 markers of the donor type and (b) degree and specificity of tolerance. Spleen cells from the CBA-BALB/c chimeras were all of the CBA H-2 type as measured by killing of cells by anti-H-2^b plus complement. These spleen cells were also specifically tolerant to BALB/c antigens; they were unable to yield anti-BALB cytotoxic cells in response to BALB/c stimulator cells, but could produce a normal anti-C57BL/6 cytotoxic response when stimulated with C57BL/6 cells. Although von Boehmer et al. (13) have been unable to detect any suppressor cells in radiation chimeras, it was still possible that suppressive phenomena might interfere in this system. If this were the case, however, such cells did not interfere in the induction of an anti-BALB/c response as shown in Fig. 4b. If suppressors did exist in these tolerant chimeric mice, then it is reasonable to assume that these cells would suppress a CBA anti-BALB/c response. Since the (BALB/c \times C57BL)F₁ stimulator cells have BALB/c determinants, any anti-BALB/c suppressors should thus suppress both the anti-BALB and the anti-C57BL cytotoxic responses. Neither response was suppressed, thus demonstrating that suppressors were not a complicating factor here.

The experiments illustrated by Figs. 4 and 6 show that anti-C57 helper cells collaborate with anti-BALB/c killer precursors. It now becomes a matter of

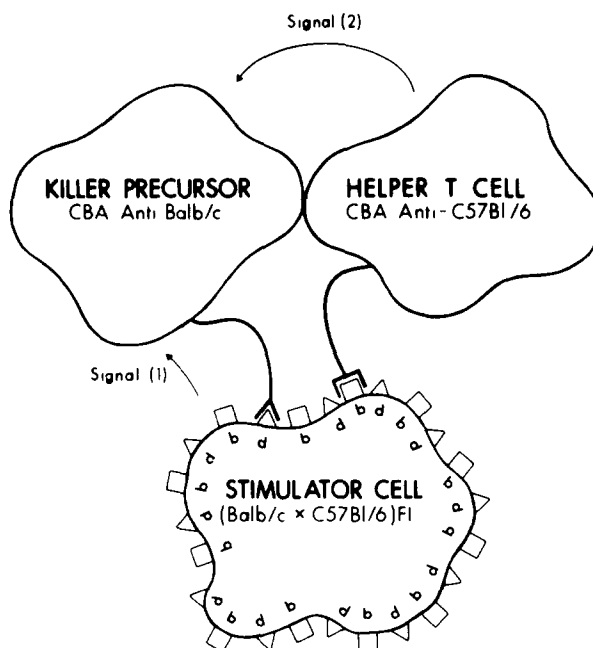


FIG. 6. A mechanism for cooperation between helper cells tolerant of BALB/c antigens and an anti-BALB/c killer precursor.

interest to determine the specificity of these helpers in more detail. As discussed in the introduction, several groups have reported evidence suggesting that the receptor specificity of the helper cells is directed either mainly or exclusively at the Ia antigens of the stimulator cell (3-5). The work of Klein and his colleagues with *H-2K* and *H-2D* end mutant mice appears to be at variance with this (6, 7); if helper cells are involved in his system then they must be specific for antigens encoded by the *H-2D* or *H-2K* end of the *H-2* gene complex. The experiments reported here lead to the prediction that since help is required for killer generation, anti-*H-2D*-specific helpers do in fact exist. In this case the mutant protein(s) might serve as both hapten and carrier determinants. Experiments to determine the specificity repertoire of the T cells which help a cytotoxic response are in progress.

The lack of strain specificity in the helper T-cell population apparent in these experiments deserves comment since it is contrary to many reports involving both T-B and macrophage-T systems (14, 15, 26), with the exclusion of experiments that analyze unprimed humoral responses in vitro, or tetraparental or chimeric mice (11-13). There are two possible mechanisms by which allogeneic cell collaboration might proceed: (a) via normal linked recognition of hapten and carrier determinants on the stimulator cell surface or (b) via abnormal induction (27, 28) involving recognition of antigenic determinants on the responder cell surface. Fig. 7 illustrates these possible mechanisms. The most convincing argument in favor of the first possibility, which predicts that the allogeneic helper effect is antigen specific, is the fact that BALB/c spleen cells do not provide help for CBA thymocyte precursors. In contrast cells of other *H-2*

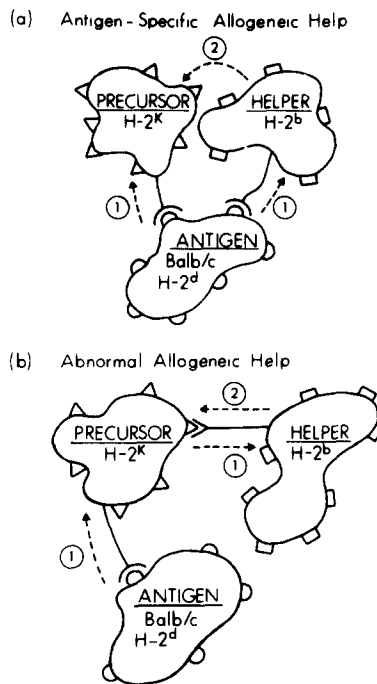


FIG. 7. Possible mechanisms for cooperation between histoincompatible helpers and killer precursors.

haplotypes help very efficiently. BALB/c spleen cells are tolerant of BALB/c antigens and therefore should be unable to help an anti-BALB/c response; they are not, however, tolerant of CBA antigens (on the responder cell) and should therefore be able to deliver an abnormal signal if that were the mechanism by which allogeneic helper cells collaborated. The lack of helper activity in BALB/c spleen cell populations strongly suggests that the allogeneic cells cooperated in an antigen-specific manner strictly comparable to the mechanism by which syngeneic cells cooperate. Such a result is not surprising if one considers that both the precursor and the helper cell populations are derived from normal mice. They are therefore presumably unprimed, although it might reasonably be expected that some form of environmental priming might be required to induce the production of differentiated T cells capable of helping the cytotoxic cell precursor. Experiments are in progress to investigate the mechanism by which allogeneic cells cooperate to yield killer T cells.

The mechanism by which the help for killer precursors occurs is not known. Two possible modes of action can be imagined: (a) a two-lymphocyte interaction in which the irradiated helpers act on the killer precursors probably via an accessory cell or (b) sequential interactions in which the irradiated helpers act indirectly on the killer precursor by first inducing a helper cell precursor in the thymocyte population to clonally expand and produce help for the killer precursor also present in the thymocyte population. To differentiate between these alternatives is experimentally difficult. An indirect mode of action seems most likely based on the fact that as one dilutes the thymocyte population, a sharp

drop in the generation of cytotoxicity occurs which cannot be due to an absence of reactive precursors. This implies that at least one other cell type which is required for a successful collaborative event is resident in the thymus. It is unlikely that this cell is an accessory cell since the cultures contain a large number of irradiated spleen cells which are a rich source of accessory function (29). Furthermore, addition of splenic adherent cells to cultures does not enhance the response at any cell dose. (L. Baum, personal communication). The possibility that the splenic helper cells collaborate indirectly does not alter the interpretation of any of the experiments. Thus, if collaboration occurs via a thymocyte helper precursor then the associative recognition and the ability to cooperate across allogeneic barriers are properties of T helper-T-helper precursor (for killers) interaction rather than of a T-helper-T-killer interaction. An experimental system is being set up to resolve this point.

Summary

Thymocytes cultured with irradiated, allogeneic stimulator cells yield no cytotoxic effector cells after a period in culture. If, however, a population of irradiated spleen cells syngeneic to the responder cells are added to these cultures, cytotoxicity is generated. The helper activity present in the irradiated syngeneic spleen cells was found to be mediated by a cell bearing theta antigens. Furthermore, it was found to be antigen specific; helper cells which were tolerant of the stimulator cell antigens were unable to help the thymocyte responder cells, although these tolerant cells did contain helpers specific for a third party antigen. These experiments are consistent with a requirement for associative recognition of linked determinants in the induction of killer precursors which is thus strictly analogous to the induction of B-cell precursors via collaboration with helper T cells. In more extensive studies, it was found that histoincompatible helper cells ($H-2^b$, $H-2^p$, $H-2^o$) were able to help a cytotoxic T cell ($H-2^i$) response to a third party stimulator cell antigen ($H-2^d$); that is, the helper T cells which interact with cytotoxic T-cell precursors are not strain specific. It seems likely that the histocompatible helper cells induce killer precursors in an antigen-specific cooperation event similar or identical to normal syngeneic cooperation.

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