

IN VITRO GENERATION OF ANTIGEN-SPECIFIC HELPER T CELLS THAT COLLABORATE WITH CYTOTOXIC T-CELL PRECURSORS*

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Originally, T-T collaboration was thought of in terms of synergy between thymocytes and lymph node cells but the functional contributions of each were not characterized (1, 2). The participation of two collaborating cell types in the induction of a cytotoxic response was also shown by later work demonstrating that the cytotoxic T precursor had Ly2,3 antigens on its surface and was apparently specific for H-2K and D region encoded determinants although the helper/amplifier cell had Ly1 antigen and appeared to be specific for determinants encoded in the I region of the H-2 gene complex (3). Even at this level of sophistication, however, the T-T collaboration events being observed reflected mainly an amplification effect rather than an absolute requirement for antigen-specific helper T cells which has been clearly demonstrated for B-cell induction in response to T-dependent antigens (4, 5).

Work from this laboratory has demonstrated such a requirement for help in the induction of cytotoxicity. Thymocytes are unable to generate cytotoxicity in response to allogeneic stimulator cells. However, in the presence of irradiated normal syngeneic spleen cells, thymocytes respond to allogeneic stimulator cells to yield a specific cytotoxic response (6). These radioresistant splenic helper T cells are antigen-specific but are not strain-specific because they appear to help across an allogeneic barrier. The antigen specificity of helper cells from unprimed normal spleen was demonstrated by showing that a mouse made tolerant to one alloantigen could not produce helper cells for that alloantigen but could still provide a source of help for a second unrelated alloantigen. While this system demonstrated that cell cooperation was an absolute requirement for the generation of cytotoxicity, it was not sufficiently flexible to answer questions about the specificity of the collaboration event. The ability to prime helpers in vitro to produce cell populations with high-specific activity would provide a means of characterizing the positive regulation of cytotoxic T-cell induction. It would also allow analysis of the regulatory events which control helper T-cell induction.

We have developed a system in which antigen-specific helper T cells are stimulated in vitro by culturing normal spleen cells with irradiated allogeneic spleen cells. The kinetics of helper production, the characteristics of the cell population mediating the helper effect, and the specificity of the helpers generated in vitro are examined in this paper.

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Materials and Methods

Mice. CBA/CaJ, BALB/cCr, C3H·SwSn, and (BALB/c × C57Bl/6) F_1 mice were obtained from the University of Alberta animal breeding facility. C57Bl/6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice of both sexes were used. Thymus cells were obtained from mice 5–6 wk of age and spleen cells were from mice 8–14 wk of age.

Basic Experimental Plan. Helper cells were generated in Marbrook acrylamide tissue culture vessels (rafts) (7, 8). Helper cells obtained from rafts (first-step cultures) were transferred to a second culture (second-step cultures) to assay the amount of help produced by measuring the ability of first-step cells to help a cytotoxic response by thymocyte killer presursors. The second-step cultures utilized either rafts or "V"-bottom, 96 well microtiter trays; most of these experiments have been done in both culture systems with similar results. When rafts were used, cultures were set up in triplicate. When microtiter trays were used, four to six replicate cultures of each group were prepared.

Cell Preparation and Culture. All cells used were collected aseptically in Leibovitz medium (L-15, Grand Island Biological Co., Grand Island, N.Y.). Stimulator cells for second-step cultures were preincubated for 90 min at 37°C as described by Lafferty et al. (9). Stimulator cells for first-step cultures were not preincubated because preincubation did not enhance the helper activity (unpublished results). All suspensions of stimulator cells were given 1,500 rads of γ -irradiation from an Atomic Energy of Canada Limited gamma cell 40 which contained cesium 137. All cell populations were washed and resuspended in minimal essential medium (F-15, Grand Island Biological Co.) + 10^{-4} β -mercaptoethanol, 10% fetal calf serum (FCS),¹ and 50 μ g/ml gentamicin (Microbiological Associates, Walkersville, Md.) for use. The batch of FCS was not important in determining the magnitude of the cytotoxic response observed (unpublished observations).

First-Step Cultures. In most experiments, first-step cultures contained 8×10^6 CBA/CaJ responder spleen cells and 8×10^6 irradiated BALB/cCr stimulator spleen cells. Cells were cultured for varying periods of time, harvested, and irradiated with 1,500 rads before the assay for helper function in second-step cultures. Except for initial experiments where the kinetics of helper cell generation were being studied, all first-step cultures were incubated for 2 days at 37°C in 10% CO₂-air. Viability of these cultures at harvest was nearly always greater than the number of responder cells originally put in culture.

Second-Step Cultures. When acrylamide rafts were used for second-step cultures, each raft contained $1-4 \times 10^6$ CBA thymocyte responder cells, 1×10^6 irradiated first-step culture cells (unless otherwise indicated) or 8×10^6 normal CBA spleen cells as a source of helper cells; the number of irradiated allogeneic stimulator cells was adjusted to bring the total number of irradiated cells to 16×10^6 per raft (15×10^6 or 8×10^6 stimulator cells). When microtiter trays were used for second-step cultures (0.2 ml/culture), $1-5 \times 10^5$ thymocyte responders were cultured with 1×10^5 irradiated first-step culture cells as helpers and 1×10^6 irradiated stimulator spleen cells. Second-step cultures were incubated at 37°C in 10% CO₂-air for 5 days before assay.

The following controls were routinely done: (a) thymocytes were cultured with irradiated helper cells in the absence of stimulator cells, (b) irradiated helper cells were cultured with irradiated stimulator cells, and (c) irradiated thymocytes were cultured with irradiated helper cells and irradiated stimulator cells. These controls were always negative.

Several experiments were done to analyze the time-course of the second-step culture cytotoxic response; the peak response under the conditions used here was always at day 5 of culture with all doses of first-step cells added.

Cytotoxic T cells, assayed at day 5 of culture, were specific cells. The CBA anti-BALB cytotoxic response exhibited very little cross-reactivity when measured on EL4 target cells; the CBA anti-H-2^b cytotoxic response was somewhat cross-reactive when measured on P815 target cells (10–15% cross-reaction). Similar results have been published by others (12, 17).

Culture and Preparation of Target Cells. P815 mastocytoma and EL4 leukemia lines were maintained in vitro as described previously (6). Culture and labeling techniques are described in detail elsewhere (6, 8). All assays were done in V-bottom microtiter wells which contained 1×10^5 ⁵¹Cr-labeled target cells per well.

¹ Abbreviations used in this paper: FCS, fetal calf serum.

Culture Harvest and Cytotoxicity Assay. These methods have been described in detail elsewhere (6, 8). When rafts were used for step-two cultures, cells were resuspended and all cells from one group were pooled, washed, and resuspended. Cultures were then diluted and assayed at 1/10, 1/30, or 1/60 of a culture per assay well (0.1 ml vol). When second-step cultures were done in microtiter trays, the medium was decanted by suction and each culture was resuspended in 0.1 ml of medium in situ. The appropriate ^{51}Cr -labeled target cells (1×10^5) were added in 0.1 ml vol, and cells were incubated at 37°C in 10% CO_2 -air of 4 h. The top 0.1 ml was then removed and the number of cpm was determined. Total release of ^{51}Cr was determined by incubating labeled targets with 0.05% Triton-X-100 detergent, and spontaneous release was determined by incubating target cells with medium. Results are expressed as:

$$\text{percent specific release} = \frac{\text{sample (cpm)} - \text{spontaneous release (cpm)}}{\text{detergent release (cpm)}} \times 100.$$

In the figures and tables, the cytotoxicity observed has been expressed as percent-specific target cell lysis per fraction of a culture rather than as a function of the number of viable cells. This is for two reasons: (a) the number of viable cells in a culture does not correlate with the amount of cytotoxicity (8), and (b) the experiments always involve comparisons between groups where the cytotoxicity of a constant number of responder cells, which have been cocultured with various accessory cell populations, is measured. This method requires the least manipulation of raw data, is the most direct way of measuring the killers derived from a given number of precursor cells, and is a means of expressing data which is routinely used in the literature on humoral immune responses in vitro. A representative experiment which includes viable cell counts has been detailed in Table I. When second-step cultures were performed in microtiter trays, the whole culture was assayed and viable counts were not done.

Anti-Theta Serum Preparation and Treatment. AKR anti-CBA theta serum was prepared by procedures previously described (6, 8). Lymphocytes to be treated were washed and resuspended in a 1/6 dilution of either normal mouse serum or anti-theta serum and were incubated at 37°C for 30 min. After centrifugation, the cells were resuspended in absorbed rabbit complement (1:16) (10) at 37°C for 45 min.

Results

Generation of Helper Cells In Vitro and Time-Course of Helper Cell Production. CBA spleen cells which have been cultured for 2 days in the presence of irradiated BALB/cCr stimulator cells yield a population of helper cells which are eight times more active than the helper cells obtained from uncultured spleen cells (Fig. 1). A time-course was done to determine the optimal time for the generation of helper cells in first-step cultures. In experiments where normal spleen cells or cells cultured for 1 day helped only marginally, cells cultured with antigen for 2 days before irradiation and inclusion in second-step cultures were extremely efficient helpers (Fig. 1, Table I). Cells cultured for 2 days with irradiated syngeneic spleen cells did not help in second-step cultures (data not shown). The results obtained with helper cells from first-step cultures harvested on day 2 indicated that when help was assayed in polyacrylamide raft second-step cultures, the optimum number of helper cells per second-step culture was 1×10^6 cells (Table I). When second-step cultures were set up in microtiter trays, the optimum number was 5×10^4 helper cells/well (Fig. 2). In other experiments, the optimal dose was 1×10^5 first-step cells/well. Although initial experiments indicated a sharp drop in helper cell activity on day 3 of culture (Table I, Fig. 1), experiments which included a comprehensive range of helper cell numbers indicated that at day 3 of culture a considerable increase in helper cell activity had in fact occurred (Fig. 2). Thus, only 5×10^3 helper cells (in microtiter trays) gave optimal activity after 3 days in culture. This represents a 10-fold increase in the activity of helper cells. The cytotoxicity generated by the collaboration between helper cells harvested at day 3 of

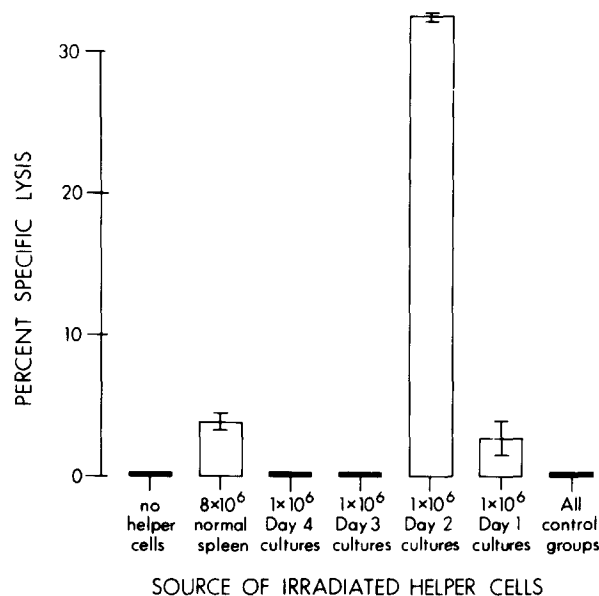


FIG. 1. Time-course of the generation of helper cells in vitro. 1×10^6 CBA thymus (H-2^k) cells were cultured in acrylamide rafts for 5 days. They were cultured with 1×10^6 CBA helper cells (H-2^k) which had been primed in vitro for the indicated time periods, or 8×10^6 normal CBA spleen cells (H-2^k). Appropriate numbers of irradiated BALB/cCr spleen cells were added as stimulators. 4-16 replicate cultures were pooled in groups of two, and three dilutions of each group were tested for cytotoxicity. The highest concentrations produced specific lysis values that were in the linear portion of the curve derived from plotting the percent specific ⁵¹Cr release versus the dose of killer lymphocytes and were therefore used in this figure. Bars represent the standard deviation between groups. The column labeled all control groups represents groups in which the irradiated helper cells were tested with either irradiated thymocytes plus irradiated stimulator cells, or with only irradiated stimulator cells. These controls were always negative. Assay was on 10^5 ⁵¹Cr-labeled P815. Detergent lysis = $4,105 \pm 73$; spontaneous release = 449 ± 20 . Machine background of 99 ± 6 cpm has not been subtracted.

culture and cytotoxic precursors was always less than the cytotoxicity obtained with cells from cultures harvested at day 2, although more cells were required for the helper population present in 2-day cultures to generate a response. This suggests that at day 3 of culture, helper cells were more frequent but less efficient than helper cells from cultures harvested at day 2.

Helper Cells Primed In Vitro are Derived from Theta-Positive Spleen Cells. Spleen cells treated with anti-theta serum and complement before cell culture were incapable of generating helper cell activity. Precursor cells treated with normal mouse serum and complement were able to produce efficient helper cells (Fig. 3).

Differentiated Helper Effector Cells Bear Theta Antigen. Treatment with anti-theta serum and complement is equally effective when differentiated helper cells that have been generated in first-step cultures are examined (Table II). Nearly all helper activity observed when cultures treated with normal mouse serum plus complement are irradiated and added to second-step thymocyte cultures is removed by treatment with anti-theta serum and complement. Removal of A cells from the first-step cell population seemed to provide enhanced helper activity for the cytotoxic response of thymocytes (Table II). It should be mentioned that these cultures did contain β -mercaptoethanol (BME) and therefore this does not indicate that cytotoxicity can be

TABLE I
Time-Course of Helper Cell Generation in First-Step Cultures

Source and number of irradiated helper cells		Percent-specific ⁵¹ Cr release fraction of a culture assayed			
		1/10	1/30	1/60	Viable cells/culture
None		0	0.2	0	4 × 10 ⁴
Normal spleen	8 × 10 ⁶	7.7	2.6	1.7	3.6 × 10 ⁵
Day-2 cultures	1 × 10 ⁶	22.6	9.3	4.0	1.6 × 10 ⁵
	1 × 10 ⁵	5.2	2.6	1.3	3.6 × 10 ⁵
	1 × 10 ⁴	0.6	0.4	0.1	4 × 10 ⁴
Day-3 cultures	1 × 10 ⁶	1.6	0	0	6 × 10 ⁴
	1 × 10 ⁵	3.3	1.9	1.4	1 × 10 ⁵
	1 × 10 ⁴	0.8	0	0.2	6 × 10 ⁴
Day-4 cultures	1 × 10 ⁶	0	0.5	0.7	2 × 10 ⁴
	1 × 10 ⁵	0.1	0.3	0.2	4 × 10 ⁴
	1 × 10 ⁴	0.9	0.3	0	8 × 10 ⁴
Day-5 cultures	1 × 10 ⁶	1.2	0	0	2 × 10 ⁴
	1 × 10 ⁵	1.3	0.1	0	4 × 10 ⁴
	1 × 10 ⁴	0	0	0	4 × 10 ⁴
Day-2 cultures*	1 × 10 ⁶	0.4	0.3	0.2	2 × 10 ⁴

1 × 10⁶ CBA thymocytes were cocultured in acrylamide rafts with 8–16 × 10⁶ irradiated BALB/c spleen cells and the indicated number and kind of helper cell populations. These second-step cultures were assayed for cytotoxicity on day 5 of culture on P815. Detergent release = 7,117 ± 752; spontaneous release = 624 ± 42. First-step raft cultures contained 8 × 10⁶ CBA spleen cells + 8 × 10⁶ irradiated BALB/c spleen cells; these were cultured for varying periods of time as indicated in the table. Viable cell recoveries from first-step cultures were as follows: day 2 = 8.3 × 10⁶ cells/culture; day 3 = 23 × 10⁶ cells/culture.

* These cultures contained irradiated thymocytes, irradiated stimulator cells, and irradiated helper cells. In the absence of help, thymocyte responder cells survive poorly. In the presence of helper cells (day 2 first-step cultures) they survive. This does not represent a feeder effect because the helper effect is specific as shown in later figures. Also note the lack of correlation between viable cell recovery and cytotoxicity (lines 2, 3, 4, 7).

generated in the absence of A cells. This observation does however strengthen the evidence which indicates that the helper cell in this system is indeed a T cell and not an adherent cell which has passively acquired theta antigens.

These results show that the helper cell generated in vitro is derived from a theta-positive precursor cell, bears the theta antigen on its surface, and is probably not an adherent cell. This indicates that the major proportion of helper activity generated during culture for 2 days with alloantigen is due to the activities of a T cell.

Helper Cell Precursors and Effectors are not Adherent to Nylon Wool. Spleen cells that had been passed through a nylon wool column (21) retained their ability to generate helper cell effectors after a 3 day culture period. Equally efficient helper populations were generated from nylon wool-passed or untreated precursor populations if one takes into account the enrichment factor of threefold after passage of cells through nylon wool (Table III). Cells passed through nylon wool after a 3 day culture period retained their ability to help but in this experiment appeared to be somewhat less efficient than were untreated helper effector cells. This was not always the case.

Helper Cell Precursors are Antigen Specific. The helper cell precursor which is stimulated to divide and differentiate in the first-step cultures is antigen specific. To do

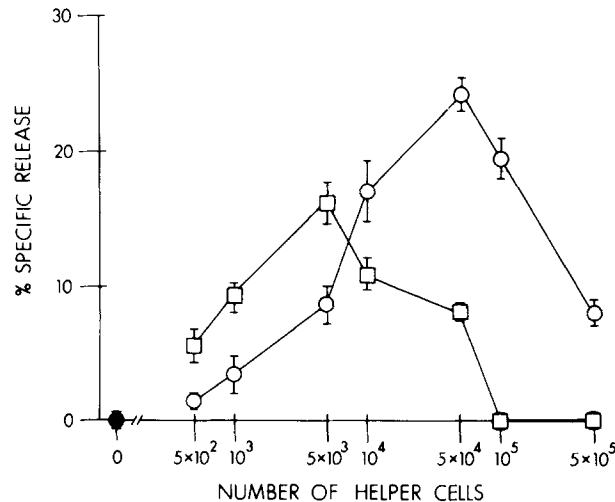


FIG. 2. Limiting dilution analysis of helper activity by cells from 2-day or 3-day cultures. 2.5×10^5 CBA thymus cells per well were cultured in microtiter trays with or without various numbers of helper cells which were primed for 2 or 3 days in vitro, and 1×10^6 BALB/cCr spleen stimulator cells. These were then incubated for 5 days. Each point represents the mean of six replicate cultures and bars indicate standard errors. ●, no helper cells added; ○, helper cells from 2-day cultures; □, helper cells from 3-day cultures. First-step cultures in which helper cells were generated contained 8×10^6 CBA spleen cells as helper precursors and 8×10^6 irradiated BALB/c stimulators. Viability in cultures harvested at day 2 was 13×10^6 cells per culture. Viability in cultures harvested at day 3 was 14×10^6 cells/culture. Assay of second-step cultures was on P815. Detergent release = $3,416 \pm 131$; spontaneous release = 291 ± 12 . The inhibitory activity seen at high cell numbers of 3 day first-step cells is not due to a shift in the time of optimum killer cell generation in the second-step cultures (20).

this, (CBA \times BALB/cCr) F_1 spleen cells were stimulated in first-step cultures by irradiated (C57Bl/6 \times BALB/cCr) F_1 spleen cells. In this particular experimental situation, if the helper cell precursor is antigen specific, we would expect the responding (CBA \times BALB/cCr) F_1 precursor cells to be incapable of producing anti-BALB/cCr helpers for reasons of self-tolerance. However, these precursors should be capable of generating a population of anti-C57Bl/6 helper cells from which the component of anti-C57Bl/6 helper cell populations, which also recognizes BALB/cCr alloantigens is absent. These newly generated (CBA \times BALB/cCr) F_1 helper effector cells were then assayed for their ability to collaborate with CBA thymocytes in response to BALB/c stimulators, which should express no determinants for recognition by the helper cell population, or to (BALB/c \times C57Bl/6) F_1 stimulator cells expressing C57Bl/6 determinants which can be recognized by the helper cells. To ensure measurement of equivalent subsets of killer precursors in both experimental conditions, only the anti-BALB/c cytotoxic response is measured. In the experiment reported in Fig. 4, an anti-BALB/cCr cytotoxic response to (BALB/cCr \times C57Bl/6) F_1 stimulators was observed (50% lysis) but there was no response to BALB/cCr stimulator cells (2% lysis). No helper cells capable of recognizing determinants expressed on BALB/c stimulator cells were detected. These results indicate that only helper cells specific for C57Bl/6 alloantigens were generated from (CBA \times BALB/cCr) F_1 precursor cells. Similar results were obtained when (CBA \times BALB/cCr) F_1 spleen cells were stimulated by irradiated C3H \cdot SwSn (H-2^b) spleen cells. Again, the only response observed

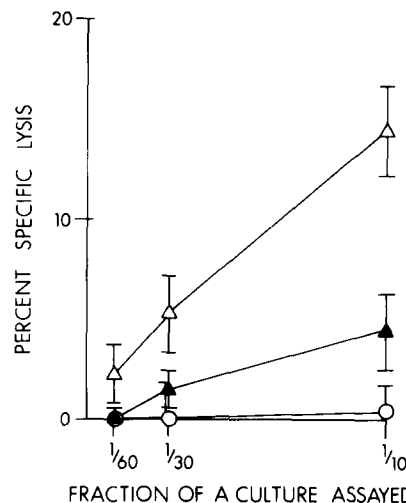


FIG. 3. Generation of helper cells from anti-theta-treated spleen cells. 8×10^6 CBA spleen cells were treated with normal mouse serum or anti- θ serum and complement before culture with 8×10^6 irradiated BALB/cCr stimulator cells for 2 days. The helper activity of these cultures was then assayed by mixing 1×10^6 CBA thymocytes with or without these helper cells, and with fresh irradiated BALB/cCr stimulator spleen cells in a second-step culture. Points represent the mean of four replicate cultures and bars indicate standard deviations. Cells which had been treated with anti-theta serum and complement were not enriched for viable cells before use in first-step cultures to avoid experimental manipulation of the natural ratio of various cell types to one another. However, equal numbers of first-step cells from each group were added to second-step cultures. Assay of second-step cultures was on P815. Detergent release = $2,277 \pm 504$; spontaneous release = 339 ± 29 . Machine background of 95 ± 8 has not been subtracted. ○, second-step cultures without added helper activity; △, helper activity from first-step cultures which contained cells treated with normal mouse serum \pm complement before culture; ▲, helper activity from first-step cultures containing cells which were treated with anti-theta serum + complement before culture.

when these helper cells were assayed for their ability to collaborate in the generation of anti-BALB/cCr cytotoxicity was found when (BALB/cCr \times C57Bl/6) F_1 cells were used as stimulator cells (52% lysis). There were no cells capable of helping a response to BALB/cCr stimulator cells. These results indicate that anti-BALB/cCr helpers are not generated from helper precursor populations which are tolerant of BALB/cCr determinants. The anti-C57Bl/6 or anti-C3H.SwSn helpers (both anti-H-2^b) which are generated in these cultures can cooperate with anti-BALB/cCr killer precursors in response to (BALB/cCr \times C57Bl/6) F_1 stimulator cells to induce an anti-BALB/cCr cytotoxic T-cell response.

When anti-BALB/cCr helpers are generated by stimulating CBA spleen cells with irradiated BALB/cCr spleen cells, anti-BALB/cCr helpers are generated and can collaborate in the induction of an anti-BALB/cCr cytotoxic response. This control shows that anti-BALB/cCr helper activity can be generated when appropriate cell combinations are used (Fig. 4a). When thymocyte responders were cultured in the absence of any helper cells, no cytotoxicity was generated in the second-step cultures (Fig. 4d). Thymocytes cultured with helper cells from each of the above first step cultures, in the absence of stimulator cells, yielded no significant cytotoxicity.

Helper Effector Cell Activity is Antigen-Specific. CBA anti-H-2^b helper cell activity generated in culture is also antigen-specific (Fig. 4). Anti-BALB/cCr (H-2^b) helpers can help a response to BALB/cCr stimulator cells (21% lysis) but anti-C57Bl/6 (H-2^b)

TABLE II
Helper Cells Primed In Vitro Bear Theta Antigens

Irradiated helper cells	Treatment	Cytotoxicity
		% <i>specific lysis</i>
None	None	1.9% \pm 0.4
CBA spleen primed to	None	27.3% \pm 5.0
BALB/c in vitro	Normal mouse serum + C'	21.1% \pm 3.8
BALB/c in vitro	Anti-theta + C'	3.7% \pm 2.8
BALB/c in vitro	A cells removed*	40.9% \pm 7.8

Second-step cultures of 5×10^5 CBA thymus (H-2^k) cells were cultured in microtiter trays for 5 days with or without 1×10^5 in vitro-primed helpers treated as indicated, and with 1×10^6 irradiated BALB/c spleen cells as stimulators. CBA spleen cells were primed in vitro in acrylamide rafts for 2 days with irradiated BALB/c spleen cells as antigen. Cytotoxicity values represent the mean of six replicate cultures \pm 1 standard deviation. Approximately 60% of the first-step cells were killed by treatment with anti-theta serum and complement. No cells were killed in the normal mouse serum + complement controls. Anti-theta-treated cells were used at the viable cell numbers recorded before treatment so as to avoid enrichment of other cell types which were unaffected by the treatment.

Based on the data expressed in Fig. 2, and on the experience of many similar experiments, the helper cell activity in untreated cell populations can be observed in as few as 1×10^4 first-step cells. Therefore, the 40% of the viable cells left after anti-theta treatment, when added to second-step cultures, is well within the range of detectable helper activity if the helper cell did not bear theta antigen. Removal of 90% of the viable cells by a nonspecific means would still leave detectable helper activity in an aliquot of 1×10^5 cells.

* Adherent cells were removed by allowing them to adhere to a plastic Petri dish for 30 min. Nonadherent cells were harvested by gentle rocking of the dish and removal of loose cells into a tube. Approximately 30% of the total viable cells were lost by this treatment. The harvested nonadherent cells were used at the cell concentration recorded before treatment since enrichment of nonadherent cells might obscure any possible decreased helper activity due to loss of adherent cells.

TABLE III
Helper Cell Precursors and Effectors Do Not Adhere to Nylon Wool

Helper cell source	None	First step cells $\times 10^{-3}$ % specific ⁵¹ Cr release (SE)			
		30	10	3	1
Untreated	6 (1)	33 (7)	42 (7)	31 (6)	8 (1)
Passed through nylon wool prior to first step culture		28 (5)	29 (4)	39 (7)	30 (9)
Passed through nylon wool after first step culture		—	27 (5)	21 (3)	8 (3)

Cells which had been either passed through a nylon wool column (21) or left untreated were cultured for 3 days with irradiated BALB stimulator cells in first-step culture. In this step, 30% of the cells were recovered after treatment. Cells harvested from untreated first-step cultures were then either passed through a nylon wool column or left untreated. 50% of the cultured cells were recovered in the column effluent. Cell numbers added to cultures were based on the actual viable count in the nylon column effluent. Second-step cultures were in microtiter trays containing 3×10^5 CBA thymus cells and 5×10^5 irradiated BALB stimulator cells with or without first-step cells. All groups consisted of 6-12 replicate cultures.

helpers or anti-C3H \cdot SwSn (H-2^b) helpers cannot (0.2% lysis). These helper cells are competent, however, because they are capable of helping the anti-BALB/cCr cytotoxic response to (BALB/cCr \times C57Bl/6)F₁ spleen cells.

The ability of various populations of helper cells to collaborate with anti-C57Bl/6

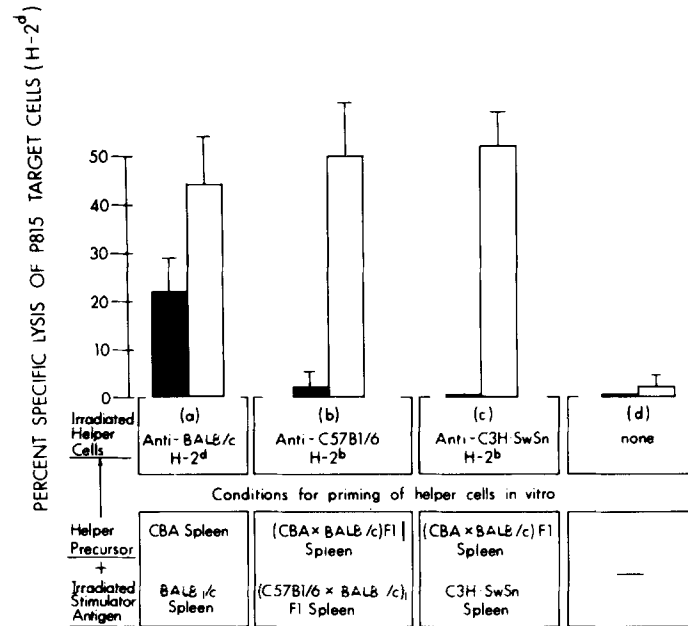


FIG. 4. Helper cell precursors are antigen specific. 3×10^5 thymocytes were cultured in microtiter trays with 1×10^5 helper cells and 1×10^5 irradiated stimulator cells as indicated in the figure. Results are expressed as the mean of six replicate cultures ± 1 standard deviation. Assay was on P815. Detergent release = $5,067 \pm 264$; spontaneous release = 599 ± 28 . Machine background of 98 ± 14 has not been subtracted. Cultures for the generation of help contained 10×10^6 of the indicated responder cells and 10×10^6 of the indicated irradiated stimulator cells. Viability in first-step cultures at day 2 was (a) 6.8×10^6 cells/culture, (b) 7×10^6 cells/culture, (c) 10×10^6 cells/culture. Thymocytes which had been cultured for 5 days with irradiated helper cells from each of the first-step cultures, in the absence of stimulator cells, and then assayed for anti-BALB/c cytotoxic response yielded 0–2% specific release of ^{51}Cr . Irradiated helper cells cultured with irradiated stimulator cells yielded no specific release. ■, response to BALB/c stimulator cells; □, response to (BALB/c × C57Bl/6)F₁ stimulator cells.

cytotoxic T precursors was also tested (Fig. 5). In the positive control group (Fig. 5, open columns), helper cell precursors were stimulated with (BALB/cCr × C57Bl/6)F₁ cells to generate a population of helper cells which contains both anti-BALB/cCr and anti-C57Bl/6 specificities. Helper cells primed to both BALB/cCr and C57Bl/6 antigens are capable of helping both a CBA anti-C57Bl/6 cytotoxic response to C57Bl/6 stimulator cells as measured by lysis of ^{51}Cr -labeled EL4 (H-2^b) and a CBA anti-BALB/cCr cytotoxic response to BALB/cCr stimulator cells as measured by lysis of ^{51}Cr -labeled P815 (H-2^d) tumor cells. Helper cell precursors were also stimulated with C57Bl/6 stimulator cells to generate a population of helper cells which were primed only to C57Bl/6 determinants (Fig. 5, hatched columns). These anti-C57Bl/6 helpers were capable of helping the induction of an anti-C57Bl/6 response but were unable to help in the induction of an anti-BALB/cCr response to BALB/cCr stimulator cells. In the absence of help, no responses were observed. It is interesting that in this particular experiment, there appeared to be no component of the anti-C57Bl/6 helper population which could crossreact with BALB/cCr determinants. In experiments where helper precursor cells are not tolerant to BALB/cCr alloantigens, we might expect that some helper cells would be primed to determinants which are

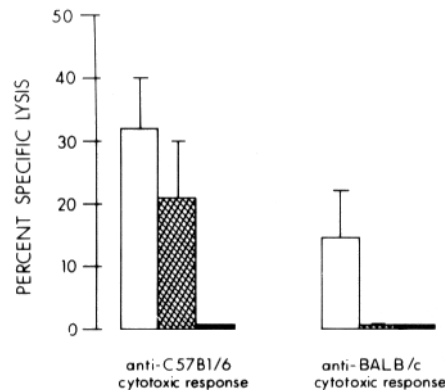


FIG. 5. Helper cell effectors are antigen specific. 5×10^5 CBA thymocyte responders were cultured in microtiter trays with 1×10^6 irradiated stimulator cells (either C57BL/6 or BALB/c spleen cells). A range of helper cell numbers was tested and the results shown are those obtained with the optimal helper cell dose. Experimental results are expressed as the mean cpm of four replicate cultures ± 1 standard deviation. Assay was on P815 for anti-BALB/c response; detergent lysis = $4,842 \pm 463$, spontaneous lysis = 340 ± 38 . The anti-C57BL/6 response was assayed on EL4: detergent lysis = $6,867 \pm 1,338$, spontaneous lysis = 690 ± 56 . Two different types of helper cell populations were used. (a) 8×10^6 CBA spleen cells + 8×10^6 (BALB/c \times C57BL/6) F_1 helper cells; open columns represent second-step cultures to which this helper cell population has been added. (b) 8×10^6 CBA spleen cells + 8×10^6 irradiated C57BL/6 spleen cells. These yield anti-C57BL/6 helper cells; hatched columns represent second step cultures to which these cells have been added. Solid columns represent second-step cultures to which no helper cells have been added.

shared by C57BL/6 and BALB/cCr. This is particularly true since both the antibody response to alloantigens (11) is extensively cross-reactive, and the killer cell response (12, and unpublished observations) also cross-reacts. In some experiments, we also observe various degrees of cross-reactive helper activity. The cross-reactive component of the helper response can only be reproducibly eliminated when helper cell precursors are tolerant to one particular set of alloantigens as in the experiment reported in Fig. 4.

Discussion

Helper cells generated in the in vitro system described here have properties similar to helpers of a B-cell response that are generated and assayed in vivo and in vitro (4, 5, 13, 14, 29). Both helper cell precursors and the differentiated helper effector cells which are obtained after a 2-day culture period bear the theta antigen. Furthermore, the helper T-cell precursor and the helper T-cell effector are antigen specific. Helper cell precursor populations that are tolerant of a particular alloantigen cannot be instructed to produce helper T cells of the tolerated specificity. Helper effectors which have been generated in response to a particular alloantigen are effective only when the relevant alloantigen is present in the second-step cultures where helper activity is assayed. When the activity of antigen-specific helper T cells generated in vitro is compared to the helper activity of normal spleen cells, the helper cells primed in vitro are approximately 10-fold more active.

The kinetics of helper cell generation require further comment. The helper activity observed after 1 day of culture is extremely low. After 2 days in culture, efficient helper activity has been generated. At 3 days of culture, cell numbers which are optimal for cells from 2 day cultures do not help; however, considerably fewer cells

(5×10^3) did provide optimal help. Other experiments which measure negative regulation of a cytotoxic response have been reported from this laboratory (20); these experiments indicate that cells capable of inhibiting the induction of a cytotoxic response appear at day 3 in first-step cultures which are identical to the cultures discussed here. In light of this, our results could be interpreted to mean that by day 3, so many helper cells have been generated in first-step cultures that they now act as inhibitors at high cell numbers. Bretscher has postulated that inhibitors of cell-mediated immunity will in fact be helper cells for humoral immunity and that too much helper signal will turn off the induction of cell-mediated immunity (15). It is possible that high levels of help may inhibit the induction of cytotoxic T-cell precursors, since sufficiently high numbers of helper cells from both 2 and 3 day cultures always inhibit the induction of a cytotoxic response. However, studies on the Ly phenotype of the regulatory cells present in 3-day cultures indicate that the major suppressive activity is due to a T cell which bears both Ly1 and Ly2 antigens while the helper cell bears only the Ly1 antigen indicating that help and suppression are carried out by two physically distinct cell populations in our system.²

Anti-theta treatment is effective in removing the helper activity regardless of whether it is performed before the generation of helpers in culture or immediately before irradiation of in vitro primed helper cells and inclusion in second-step cultures. This indicates that the help provided by cells from first-step cultures in this system derives from and is mediated by a T cell. Although most of the helper precursor activity was removed by treatment with anti-theta serum and complement, some help was still observed. This could be due to the differentiation of cells that did not possess high enough levels of theta antigen for removal by complement or it could represent a very low level of help by another cell population as suggested by Dyminski and Smith (16). The first possibility seems most likely as the helper cell precursor is not retained by a nylon wool column.

Helper precursors are antigen specific. When (CBA \times BALB/cCr) F_1 spleen cells, which do not respond to BALB/cCr alloantigens for reasons of self-tolerance, are stimulated by irradiated (C57Bl/6 \times BALB/cCr) F_1 spleen cells, only anti-C57Bl/6 helper cells are generated. If tolerance by this F_1 responder of BALB/cCr alloantigens is due to deletion of self-reactive clones of helpers, then we would interpret this result by concluding that the population of helper precursors consists of specific clones. Based on the high frequency of cells able to respond to alloantigens, it has been suggested that at least some proportion of alloantigen-reactive cells may acquire their specificity after contact with antigen, implying some variety of instructional mechanism (17). Therefore, an alternative explanation for our observation is that suppressors of self-reactivity inhibit instruction which might lead to anti-BALB/cCr activity. In terms of the specific commitment of helper precursors, this explanation says that helper precursors are not precommitted and acquire their specificity only after contact with antigen. In our particular experimental case, if helper cell specificity were acquired by an instructional type of mechanism, these anti-BALB suppressors should also prevent instruction leading to anti-C57Bl helper cell activity since the only source of antigen is a (BALB \times C57Bl) F_1 stimulator cell. The fact that we find no anti-BALB/cCr activity and yet anti-C57Bl activity remains indicates that helper cell

² A. R., Al-Adra, et al. Surface markers on the T cells which regulate cytotoxic T-cell responses. Manuscript in preparation.

precursors are committed to expression of a particular specificity before being subjected to the unknown process which leads to self-tolerance and before contact with foreign antigens. We therefore feel justified in concluding that the responder cells in first step cultures consist of a set of precommitted helper precursors expressing receptors specific for foreign antigens which in this particular case includes receptors specific for C57Bl alloantigens.

Helper cell effectors are also antigen specific in the sense that the stimulating alloantigen to which helper cells were primed in the first culture must be present in the second-step culture to observe a helper effect (Fig. 4 and 5). In the above paragraph, we have discussed the evidence which supports the idea that the induction of helper precursors is a highly specific event; this specificity is also seen in experiments which show that helper cell effectors must be activated by antigen in second step cultures. We have not yet shown specificity at the level of the subsequent helper signal. The specifically triggered helper signal might act by one of two plausible mechanisms. It might be a labile factor, or a factor which is effective only at high concentrations, and is therefore capable of signal delivery only to a killer precursor which recognizes antigen in close proximity to the helper cell (e.g. a short range factor). If this were the case, the helper signal would be seen to be highly specific. On the other hand, a specifically triggered helper cell might release a factor which was stable, or was effective at low concentration, and would therefore be capable of delivering a collaborative signal to any killer precursor cell which was recognizing antigen (e.g. a long-range factor). If this were the mechanism of collaboration, then the helper effect would appear to be nonspecific but would require a specific interaction to produce the nonspecific helper factor. Experiments are in progress to study the specificity of helper activity at this level.

The chain of events leading to a cytotoxic response in this system is still not clear. Certainly a helper T cell is required for the induction of cytotoxic T cell precursors but it is uncertain whether the helper T cell generated in our cultures interacts directly with the killer precursor or whether it acts indirectly via induction of helper cell precursors resident in the thymocyte responder population. Our preliminary evidence which indicated that helper cells generated in vitro are capable of participating in the induction of helper precursors resident in thymus lends support to the latter point. Based on evidence obtained by studies on the inductive events leading to antibody production (8), the most likely mechanism of action is one whereby differentiated helper cell effectors collaborate in the induction of helper cell precursors which then differentiate to yield more helper cell effectors. Finally, the role of macrophage-like cells in these inductive events remains to be established.

Summary

Antigen-specific helper T cells are required in the generation of cytotoxic T cells from thymocyte precursors. We have demonstrated that these alloantigen-specific helper cells can be generated in vitro and that both the quantity and quality of the helpers appear to be superior to the help obtained from unprimed spleen cells. Optimal helper cell activity is produced at day two of culture when CBA splenic helper precursors are stimulated by irradiated allogeneic spleen cells. Helper cell precursors are antigen-specific cells which cannot be instructed to express forbidden receptor specificities and bear theta antigen on their surface. The helper effectors are radioresistant, theta-bearing, and antigen-specific cells.

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