

HELPER T CELLS ARE REQUIRED FOR THE POLYCLONAL STIMULATION OF CYTOTOXIC T CELLS BY CONCAVALIN A*

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Polyclonal activation by mitogens of precursor cells to yield effector cells is commonly regarded as a result of the direct action of the mitogen on the precursor cell. Such a view predicts that isolated killer or B-cell precursors can be stimulated by mitogens to yield effector cells in the absence of any other cell type. In this series of experiments we show that the polyclonal production of cytotoxic T cells provoked by concanavalin A is dependent upon a helper T cell, indicating that even polyclonal activation involves T-cell collaboration and that the above prediction is not fulfilled.

Previous experiments have shown that thymocytes represent a population of cytotoxic T-cell precursors relatively uncontaminated by helper T cells. In these experiments it was demonstrated that an alloantigen-specific helper T cell present in irradiated spleen was required for the generation of specific cytotoxic effector cells from thymocyte precursors (1). The experiments we describe here were analogous to the above system except for the fact that the stimulator cell was replaced by the polyclonal activator concanavalin A (ConA)¹ in the generation of cytotoxic T cells. Thymocytes were used as a source of cytotoxic T-cell precursors and irradiated syngeneic spleen served as the helper cell population.

The ability of ConA to stimulate the generation of cytotoxic T cells has been described extensively (1-8) and the cytotoxicity generated is specific but polyclonal (4-7). That is, spleen cells which are tolerant to a given alloantigen are unable to produce cytotoxic T cells of that specificity when stimulated by ConA even though they produce normal levels of cytotoxicity to other alloantigens (7). Although previous workers have reported synergistic interactions between subsets of T lymphocytes in the proliferative response to ConA, the cell types participating in these interactions were not functionally defined and no studies bear on a need for cell cooperation in the generation of effector cell function by ConA (9-12). A suppressor cell can be induced in the presence of ConA that is able to nonspecifically suppress the generation of cytotoxic T cells (13) and both helper and suppressor cells for the induction of humoral responses have been produced in response to ConA (14-16). The present work represents the first demonstration that T-T collaboration is an essential component in the ConA-driven polyclonal stimulation of cytotoxic T cells. The cytotoxic T-cell precursors must collaborate with helper T cells present in irradiated normal syngeneic spleen to allow production of cytotoxicity in response to ConA.

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¹ *Abbreviations used in this paper:* A cells, adherent cells; BME, β -mercaptoethanol; ConA, concanavalin A.

Materials and Methods

Animals. CBA/CaJ and BALB/cCr mice were obtained from the University of Alberta animal breeding facilities. Thymocyte donors were always 5-wk old and spleen cell donors were 8- to 12-wk old.

Materials. ConA was purchased from Calbiochem, San Diego, Calif.

Culture Method. Cells were cultured in Marbrook acrylamide rafts as described previously (17) in the presence of 10^{-4} M β -mercaptoethanol (BME). Helper spleen cells were preincubated in medium containing BME for 90 min at 37°C before receiving 1,000 rads irradiation from a ^{137}Cs source as described previously (1).

Preparation of Adherent Cells (A Cells) and A-Cell-Depleted Cell Populations. A cells were obtained by incubating spleen cells in tissue culture dishes and harvesting the A cells. In detail, spleen cells were incubated for 30 min, loose cells were flushed from the surface and then allowed to settle for 60 min. Before collection, loose cells were removed by gently washing with medium. Phosphate-buffered saline-EDTA solution (18) was then added and the dishes were scraped with a rubber policeman to remove A cells. When cell populations depleted of A cells were required, the cells were removed by gently rocking the tissue culture dishes and pipetting off the free cells.

Assay. In all experiments reported here, cultures were harvested, resuspended in 0.6 ml and assayed at $1/6$, $1/18$, and $1/36$ of a culture per assay well. All assays contained phytohemagglutinin at 10 $\mu\text{g}/\text{ml}$ as a agglutinating agent to reveal killer cells of all specificities (4, 19). Therefore, all experiments reported here measure polyclonal killing rather than a specific subset of killers. Cultures were assayed on ^{51}Cr -labeled P815 tumor cells as targets as has been described previously (1). The percent specific lysis was calculated according to the formula:

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

Results

Activation of Cytotoxic T Cells by ConA Requires a Helper Cell. CBA thymus cells cultured in the presence of ConA do not yield cytotoxic effector cells although spleen cells cultured under the same conditions yield good polyclonal cytotoxicity (Table I). When thymus cells are cocultured with syngeneic irradiated spleen cells as well as ConA, a substantial polyclonal cytotoxic response occurs (40% lysis by one-sixth of a culture; killer to target ratio, 4.7:1). The cytotoxicity is most probably derived solely from the thymus cells as cultures containing irradiated thymus, irradiated spleen, and ConA do not produce cytotoxic effectors (Table I, line 5). Cytotoxicity was found to be optimal at day 4 of culture (Table II) at a dose of 2 $\mu\text{g}/\text{ml}$ ConA (Table III).

The Helper Function is T-Cell Dependent. The cell present in irradiated spleen and required for ConA-mediated activation of cytotoxicity is most likely to be a T cell or an adherent cell. To determine the nature of this cell the susceptibility of the activity of the irradiated spleen cells to either normal mouse serum and complement (C) or to anti-theta serum and C was determined. Fig. 1 shows that anti-theta serum-treated spleen cells were unable to help the generation of a thymocyte cytotoxic response. In contrast, normal mouse serum-treated spleen cells were able to help as well as untreated spleen cells. A second experiment of this type showing the same result is presented in Table VIII.

To further characterize the helper activity, A cells were tested for their ability to induce a cytotoxic response by thymocytes (Table IV). Normal spleen cells helped the generation of cytotoxicity by thymocytes yielding up to 66% cytotoxicity while the A cells derived from 8×10^6 spleen cells did not help the response at all (Table IV, lines 3 and 4). Similarly, if A cells were required for the helper

TABLE I
ConA Activation of Cytotoxic T Cells Requires Radioresistant Helper Cells

Cytotoxic precursors	Irradiated helper Cells	Con A	Cytotoxicity: cpm released (% specific release)			Viable cells × 10 ⁻³ per culture
			Fraction of a culture assayed			
			1/6	1/18	1/36	
CBA thymus	-	+	295 ± 4	301 ± 36	302 ± 14	3.7
CBA thymus	CBA spleen	-	267 ± 13	280 ± 13	277 ± 25	1.1
CBA thymus	CBA spleen	+	1,204 ± 75 (40.5)	938 ± 35 (29)	646 ± 42 (16.6)	28
CBA thymus*	CBA spleen	+	295 ± 8	285 ± 36	314 ± 8	4.5
CBA spleen†	-	+	1,727 ± 76 (28.2)	1,412 ± 45 (21.2)	1,281 ± 39 (18.3)	36.2

5 × 10⁶ thymus cells were cultured with or without 2 μg/ml of ConA and with or without 10⁷ irradiated CBA spleen cells and assayed 4 days later on 10⁶ ⁵¹Cr-labeled P815 target cells in the presence of 10 μg/ml of PHA. Detergent lysis, 2,316 ± 34; spontaneous lysis, 266 ± 18. Machine background of 94 ± 11 has not been subtracted.

* Thymocytes were irradiated with 1,000 rads.

† 10⁷ CBA spleen cells were cultured with 2 μg of ConA. Assay was on day 4. Detergent release, 4,527 ± 103; spontaneous release, 451 ± 30.

TABLE II
Time-Course of Cytotoxic T-Cell Development Activated by ConA

	% ⁵¹ Cr release by 1/18 of a culture	
	Day 3	Day 4
Thymocytes (without ConA)	-	0.6
Thymocytes plus irradiated spleen (without ConA)	-	0
Thymocytes	5.9	2.3
Thymocytes plus irradiated spleen	49.7	62.7
Irradiated spleen	-	4.6

5 × 10⁶ CBA thymocytes were cultured with or without 10 × 10⁶ irradiated CBA spleen cells plus 2 μg/ml of ConA. Assay was on 10⁵ ⁵¹Cr-labeled P815 target cells in the presence of 10 μg/ml PHA. Detergent lysis: day 3, 3834 ± 114; day 4, 7,533 ± 600. Spontaneous lysis: day 3, 368 ± 14; day 4, 734 ± 22. Machine background of 98 ± 10 has not been subtracted.

effect, depletion of A cells should prevent the spleen cells from exerting help. This was not the case. To assess the A-cell dependence of the response, A-cell-depleted thymus cells were cultured with irradiated A-cell-depleted spleen cells or irradiated normal spleen plus ConA. In both cultures (Table IV, lines 5 and 6) substantial cytotoxicity was observed (35% lysis by 1/6 of a culture). This indicates that within the limits of these experiments A cells are not required for a ConA-activated cytotoxic response. It should be noted that all cultures contained BME which could have potentiated the function of any remaining A cells, thereby making the response appear to be A-cell independent.

Strain Specificity of the Helper Cell. Since in some systems helper activity has been shown to be allogeneically restricted, it was of interest to determine the strain specificity of the T cells required to help ConA-driven cytotoxic responses. BALB/c thymus cells were cultured with irradiated BALB/c spleen, CBA spleen, or (BALB × C57BL/6)F₁ spleen in the presence of ConA (Table V). As was the case with CBA thymus cells, BALB/c thymus cells were unable to differentiate to cytotoxic effector cells in the absence of help. In the presence of syngeneic helper cells, a response of 32% lysis was observed in response to ConA (Table V, lines 2 and 4). Both CBA and (BALB × C57)F₁ spleen cells were

TABLE III
Dose of ConA Required for Maximum Activation of Cytotoxic T Cells

	% ⁵¹ Cr released by 1/18 of a culture		
	Dose of ConA		
	0.2 μg	2 μg	20 μg
Thymocytes	0	5.2	0
Thymocytes plus irradiated spleen	0	32.5	0

4×10^6 BALB/c thymocytes were cultured with or without 10×10^6 irradiated BALB/c spleen cells plus the indicated dose of ConA. Assay was on day 4 on 10^5 ⁵¹Cr-labeled P815 target cells plus 10 μg/ml PHA. Detergent lysis, $7,454 \pm 327$; spontaneous lysis, 768 ± 43 . Machine background of 119 ± 10 has not been subtracted.

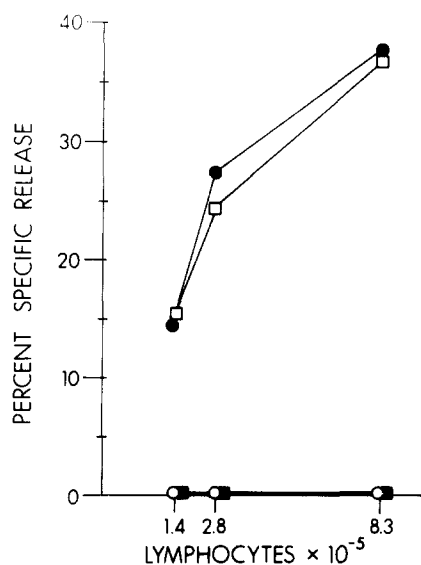


FIG. 1. The helper activity for ConA-generated cytotoxicity is T-cell dependent. 5×10^6 CBA thymus cells plus ConA (2 μg/ml) were cultured with irradiated CBA spleen cells which had been treated in various ways before culture. Assay was at day 4 on 10^5 ⁵¹Cr-labeled P815 target cells in the presence of PHA. (O-O), Thymus cells plus ConA; (●-●), thymus cells, ConA, and irradiated normal spleen cells; (□-□), thymus cells, ConA, and irradiated normal mouse serum plus C'-treated spleen cells; (■-■), thymus cells, ConA, and irradiated anti-theta serum plus C'-treated spleen cells.

capable of efficient help in the generation of a polyclonal cytotoxic response by BALB/c thymocytes (30–52% lysis), indicating a lack of allogeneic restriction in the cooperation event. This is strictly analogous to the lack of restriction displayed by the alloantigen-specific helpers for killer cells observed previously (1).

Are the Helper T Cells Required for a ConA Response Specific or Are They Polyclonal? We decided to determine whether the helper T cells were specific for ConA or polyclonal. Since the alloantigen-specific helper effect described previously (1) was present in $4-8 \times 10^6$ spleen cells but absent in 1×10^6 spleen cells, it seemed likely that a ConA-specific helper might be of similar or lower

TABLE IV
A Cells Do Not Provide Helper Function for ConA-Induced Cytotoxicity and Depletion of Adherent Cells Does Not Remove Helper Activity

Precursors	Irradiated helpers	ConA	Cytotoxicity: % specific ⁵¹ Cr release fraction of the culture assayed		
			1/6	1/18	1/36
Thymocytes	—	+	0.8	0.2	0.6
Thymocytes	Normal spleen	—	0.1	0.5	0.8
Thymocytes	Normal spleen	+	66.1	33.6	28.2
Thymocytes	A cells	+	0.1	0.8	0.1
A-cell-depleted thymocytes	A-cell-depleted spleen	+	33.7	16.4	12.3
A-cell-depleted thymocytes	Normal spleen	+	35.1	25.5	10.8

Cultures contained 5×10^6 thymocytes or an equivalent number of A-cell-depleted thymocytes and 8×10^6 irradiated spleen cells or the equivalent number of A-cell-depleted spleen cells. A cells were added as the A cells derived from 8×10^6 spleen cells. All cells were of CBA/CaJ origin. Assay was at day 3. Cytotoxicity was measured by release of ⁵¹Cr from labeled P815 target cells in the presence of 10 μ g/ml PHA. Detergent release, $6,757 \pm 831$; spontaneous lysis, 416 ± 6 . Machine background of 98 ± 13 has not been subtracted. *Nota bene*, all cultures contained 10^{-4} M BME.

TABLE V
The ConA Helper T Cell is Not Strain Specific

Responder cells	Irradiated spleen cells	ConA	Percent ⁵¹ Cr Release by 1/18 of a culture
BALB/c thymocytes	—	—	0
"	—	+	5.2
"	BALB/c	—	0
"	BALB/c	+	32.5
"	CBA	—	4.8
"	CBA	+	52
"	(BALB \times B6)F ₁	—	0
"	(BALB \times B6)F ₁	+	30

4×10^6 thymocytes were cultured with 10×10^6 irradiated spleen cells plus 2 μ g/ml ConA. Assay was at day 4 on 10^5 ⁵¹Cr-labeled P815 target cells plus 10 μ g/ml PHA. Detergent lysis, $7,454 \pm 327$; spontaneous lysis, 768 ± 43 .

frequency. Conversely, if the ConA helper cells were a population of helpers with polyclonal specificity, we might expect to see a much higher frequency of help. Fig. 2 illustrates an experiment in which various doses of irradiated normal spleen cells were added to thymus cell responders. Optimal helper activity was seen with 10^7 spleen cells per culture and a considerable degree of help was seen with 10^6 irradiated spleen cells per culture. Only minimal help was observed when 10^5 helper cells were added. Thus the frequency of ConA helpers does seem to be higher than that of alloantigen-specific helper cells by a factor of about 4. Since a fourfold increase in helper frequency is not conclusive evidence for either of the above discussed possibilities, we attempted to increase the frequency of helper cells by various types of priming regimes (Table VI).

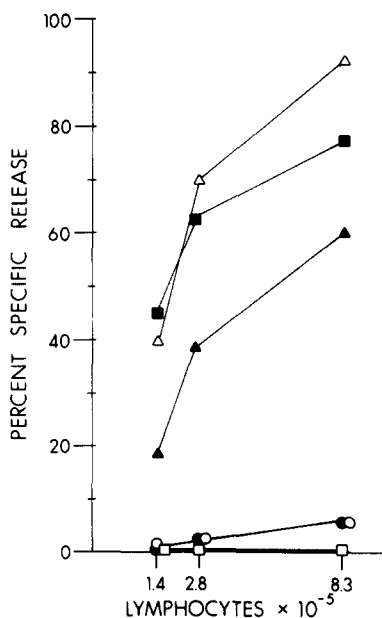


FIG. 2. The frequency of the ConA helper cell. 5×10^6 CBA thymocytes and ConA ($2 \mu\text{g}/\text{ml}$) were cultured with various doses of irradiated CBA spleen cells. Assay was at day 4 on ^{51}Cr -labeled P815 plus PHA. (●-●), Thymus cells plus ConA; (□-□), thymus cells plus 2×10^7 irradiated spleen cells, no ConA; (■-■), thymus cells, ConA, and 2×10^7 irradiated spleen cells; (△-△), thymus cells, ConA, and 1×10^7 irradiated spleen cells; (▲-▲), thymus cells, ConA, and 1×10^6 irradiated spleen cells; (○-○), thymus cells, ConA, and 1×10^6 irradiated spleen cells.

If the helper cell were specific for ConA, we expected that the priming of mice with ConA under conditions where an anti-ConA antibody response was obtained would provide a spleen cell population which was enriched for ConA-specific helper T cells. This regime did not yield enriched helper activity for ConA-driven cytotoxicity however (Table VI, line 3). Other methods of priming with ConA over a wide dose range ($1\text{--}100 \mu\text{g}/\text{mouse}$) and at two time points after priming were equally unsuccessful in elevating the level of the helper cells (Table VI, lines 5, and 7-9).

Since the helper cell did not appear to be specific for ConA, we then primed mice in ways known to produce an enriched population of helper cells specific for sheep erythrocytes (SRBC). It was reasoned that if the helper cell population were polyclonal, then increasing the number of anti-SRBC helper T cells might also increase the number of helpers for the ConA response. Activated T cells prepared by the Miller and Mitchell technique (20) were shown to provide helper T-cell activity in doses as low as 10^3 cells per culture when assayed for their ability to help an anti-SRBC plaque-forming cell response (L. M. Pilarski, unpublished data). When these cells were assayed for their ability to help a ConA-driven cytotoxic response, however, no increase in helper frequency was observed (Table VI, line 2). Other methods of priming were also unsuccessful in enriching the population of helper cells (Table VI, lines 4 and 6) although a slight increase was observed with spleen cells from mice primed 2 days previously with SRBC (Table VI, line 4).

TABLE VI
Attempts to Increase the Helper Cell Frequency

Source of helper cells	Lowest dose of helper cells/culture at which cytotoxicity was generated
1. Normal spleen	1×10^6
2. Activated T_{SRBC}	1×10^6
3. Long-term ConA-primed spleen*	1×10^6
Spleen cells taken 2 days after priming with:	
4. SRBC - 2×10^8 cells	3×10^5
5. ConA - $1 \mu\text{g}$	1×10^6
6. LPS - $1 \mu\text{g}$	3×10^6
Spleen cells taken 14 days after priming with:	
7. $1 \mu\text{g}$ ConA	1×10^6
8. $10 \mu\text{g}$ ConA	1×10^7
9. $100 \mu\text{g}$ ConA	1×10^7

In each experiment normal spleen cells and in vivo primed spleen cells were compared at four to six cell doses per culture ranging from 10^4 to 10^7 cells. The lowest dose at which cytotoxicity was generated is recorded in the table even though this dose did not yield maximal generation of cytotoxicity.

* Mice received two injections of ConA bound to SRBC at monthly intervals and a final injection of ConA bound to mouse RBC 1 mo later. Spleen cells were prepared from these mice 4 days after the last injection. Antibody titers were measured by the ability to cause agglutination of mouse RBC coated with ConA at subagglutinating concentration. Sera from mice immunized with ConA-SRBC had titers of 1/48; mice immunized with SRBC alone had anti-ConA titers of less than 1/3.

ConA Driven Thymocyte Proliferation is also Augmented by Helper T Cells. Since the differentiation of thymocytes to cytotoxic effector cells provoked by ConA is dependent upon helper T cells, it seemed likely that the proliferation induced by ConA might also be at least partially helper cell dependent. Table VII presents the results of five separate experiments in which the production of blast cells was dependent to varying degrees upon the presence of helper cells. Although the presence of ConA alone did cause some cell proliferation, the largest absolute increase in cell number occurred in the cultures containing irradiated spleen helper cells (2.4- to 15-fold increases in cell number, Table VII, lines 2 and 3). This increase was dependent on a T cell as illustrated in Table VIII where both untreated and normal mouse serum-treated spleen cells caused an approximately 10-fold increase in cell number which did not occur if the thymocytes were cocultured with anti-theta serum-treated spleen.

ConA-Driven Cytotoxicity Depends on ConA Binding to the Helper T Cell. To determine the cell type to which ConA must bind in order to stimulate generation of cytotoxicity, both precursor cells and helper cells were treated with ConA before culture, washed thoroughly, and then cultured with or without ConA (Table IX). Pretreatment of the thymocyte precursor cells with ConA had no effect on the cytotoxic response (Table IX, lines 4 and 8). In contrast, pretreatment of the irradiated spleen cells yielded enhanced cytotoxicity in both the presence and absence of ConA in the cultures (Table IX, lines 3, 6, and 7). Spleen cells pretreated with high doses of ConA and then added to untreated

TABLE VII
ConA-Stimulated Cell Proliferation is Augmented by Helper Cells

Responder cells	Irradiated syngeneic spleen cells	ConA	Blast cells per culture*				
			Day 3		Day 4		
			(a)	(b)	(a)	(b)	(c)
Thymocytes	—	—	1.1	—	2.8	0.1	0.4
"	—	+	11.7	9.4	10	4.8	12.6
"	8-10 × 10 ⁶	+	100.0	42.1	27.1	67.5	30.9
"	4 × 10 ⁶	+	43.3	—	—	—	—
"	8-10 × 10 ⁶	—	5.3	—	2.5	3.7	3.8
Thymocytes‡	8-10 × 10 ⁶	+	5.0	—	—	—	—

The absolute number of blast cells, rather than the percent blast cells, has been recorded because all of the viable cells in the 3- and 4-day cultures were large blast cells. ConA was present at 2 $\mu\text{g/ml}$.

* Each column represents a separate experiment.

‡ Thymocytes received 1,200 rads of X irradiation.

thymocytes were able to stimulate cytotoxicity even in the absence of added ConA suggesting that ConA binding to the helper cells is an important prerequisite for the generation of cytotoxic cells. If ConA were added to cultures of thymocytes and pretreated spleen, cytotoxicity was enhanced approximately sixfold (compare lysis by $1/36$ of a culture in Table IX, line 6 with the lysis by $1/6$ of a culture in line 5). Since doses of ConA lower than 2 $\mu\text{g/ml}$ do not stimulate cytotoxic cell generation, the dilution of ConA in pretreated, washed helper spleen cell preparations is such that stimulation of cytotoxicity in Table III, line 3 cannot be due to carry over of unbound ConA. This implies that ConA bound to the irradiated helper spleen cells stimulates the generation of cytotoxicity by thymocyte precursors.

Discussion

Historically, mitogens and polyclonal activators have been regarded as lymphocyte-stimulating agents which function independently of any cellular collaboration. Thus, B-cell mitogens were believed to be "T-independent" in their ability to polyclonally activate antibody synthesis and similarly T-cell mitogens were considered to be active in the absence of any collaborating T cells. Evidence has accumulated which documents a role for helper T cells in the activation of B cells by agents such as lipopolysaccharide and pokeweed mitogen (21, 22) but there have been no reports of a similar T-T collaboration occurring in the polyclonal activation of T-cell-mediated cytotoxicity by agents such as ConA. In this paper we present evidence for the essential role for a helper T cell in the generation of cytotoxic T cells from ConA-stimulated thymocytes. Thymocytes do not produce cytotoxic T cells in response to ConA. In the presence of irradiated spleen cells and ConA, however, a high level of cytotoxic activity is generated which is derived exclusively from the thymocyte population (Table I). The helper activity is dependent on a theta-bearing cell, is not provided by A cells, is present in A-cell-depleted spleen, and is sufficiently frequent to be readily detectable in normal spleen cell populations. This helper cell also

TABLE VIII
ConA-Stimulated Thymocyte Proliferation Depends on a Theta-Bearing Helper Cell

Source of help	Viable cells $\times 10^{-5}$ at day 4	Relative cytotoxic potency*
None	3.8 \pm 0.8	0
Untreated spleen	33.7 \pm 3.0	1.0
Normal mouse spleen plus C-treated spleen	39.9 \pm 16	0.7
Anti-theta serum plus C-treated spleen	2.3 \pm 0.1	0

All cultures contained 4×10^6 CBA thymocytes and 2 $\mu\text{g/ml}$ of ConA. Assay was at day 4. Helper spleen cells were all of CBA origin.

* Cytotoxicity of cultures containing treated helper cells divided by the cytotoxicity of cultures containing untreated helper cells.

appears to augment cell proliferation by 2- to 15-fold although some proliferation does occur in cultures containing only thymus cells and ConA. We do not know whether this "background" activity is due to a low level of helper cells in the thymocyte population or if it is a collaboration-independent event.

A further characteristic of this helper cell is its lack of allogeneic restriction. In this respect the ConA-dependent helper is very similar to the alloantigen-specific helper T cells which collaborate in an antigen-specific manner with cytotoxic T-cell precursors (1). The antigen-specificity of the cell which helps ConA-driven generation of cytotoxicity is still a matter for speculation. There are two possible ways a helper cell might collaborate with a precursor in the presence of ConA. Firstly, the helper cell might have antigen receptors specific for ConA and therefore recognize ConA bound to the carbohydrate moieties on the precursor cell surface. Thus a specific ConA-recognizing subset of helpers could collaborate with a precursor cell of any specificity since ConA receptors on the precursor surface would not be clonally expressed (Fig. 3a). If this were an accurate description of the collaboration then the prediction can be made that priming mice with ConA should increase the frequency of cells able to help the ConA-driven cytotoxic response.

The second possible model is that the helper effect is essentially polyclonal. That is, a helper cell of any specificity which is sufficiently differentiated to be able to deliver signals will help a cytotoxic precursor. Thus ConA would act as a stimulator and as a "glue" to bring helper and precursor cell into close proximity which would then allow signal delivery and induction (Fig. 3b). It is likely that an important aspect of this model would be nonspecific stimulation by ConA as an amplifier of both the helper signal and of precursor receptivity to that signal. The prediction which follows from the above model is that cell populations primed to a ConA-unrelated antigen (e.g., SRBC) might have an increased frequency of helper cells and certainly would not be expected to have a decreased frequency of helper cells. Conversely, if helpers were specific for ConA, then cell populations enriched for anti-SRBC helper cells might have a decreased frequency of ConA helper cells.

Experiments to test these predictions are illustrated in Tables VIII and IX and Fig. 2. The frequency of the cell which helps the ConA-driven cytotoxicity is approximately fourfold more frequent than are alloantigen-specific helpers (1) although this must be regarded as a relative frequency rather than an absolute

TABLE IX
Effect of ConA Pretreatment on the Helper and Precursor Cells

Treatment of precursor thymus cells	Treatment of irradiated spleen helper cells	ConA in the cultures	Cytotoxicity (% specific lysis)		
			1/6	1/18	1/36
1. None	None	-	0	0	0
2. None	2 μ g ConA	-	0	0	0
3. None	20 μ g ConA	-	25	19.8	14.6
4. 2 μ g ConA	None	-	0	0	0
5. None	None	+	28	14.8	11
6. None	2 μ g ConA	+	50.8	39.4	27.1
7. None	20 μ g ConA	+	55.9	44.6	32.4
8. 2 μ g ConA	None	+	28.3	17.1	10.6
9. 2 μ g ConA	2-20 μ g ConA	+	38.9	26	15.5

Cultures contained 4×10^6 CBA thymus cells and 10×10^6 irradiated CBA spleen cells. Cultures containing ConA at 2 μ g/ml are indicated in the table. Spleen helper cells were pretreated with the above doses of ConA for 90 min at 37°C before irradiation. Thymocytes were pretreated for 30 min at 37°C. Assay was on ^{51}Cr -labeled P815 plus PHA. Detergent release, $4,230 \pm 190$; spontaneous lysis, 543 ± 17 . Machine background of 98 ± 7 has not been subtracted.

value. Several priming regimes were tested in attempts to alter the frequency of the ConA helper cell. Mice were primed with ConA at various doses and at various times after priming were tested for the content of splenic helper activity. In no case did we observe an enriched frequency of helper cells. The experiment depicted in Table VI, line 3 is particularly interesting as in this case the ConA-primed mice had anti-ConA antibody indicating that an immune response directed against ConA antigenic determinants had occurred. Since ConA does not generally act as a B-cell polyclonal activator, it is reasonable to assume that if ConA-specific helper T cells exist, then they should be enriched in ConA-immune mice. In spite of the unequivocally primed state of these mice, no increase in the frequency of ConA helpers was detected. Thus, the prediction of model 1 was not fulfilled. The prediction of model 2, that mice primed to an unrelated antigen might have increased levels of helper activity, was tested by priming with SRBC in various ways known to induce anti-SRBC helper activity. In some of these experiments a slight increase in helper frequency was observed. However, when activated T_{SRBC} were prepared by the method of Miller and Mitchell (20), a technique which might be expected to produce a cell population which is heavily enriched for helper T cells, no enrichment was observed in most experiments although in one experiment a small increase in the frequency of ConA-dependent helpers was observed. Perhaps a more important observation is that activated T_{SRBC} showed no decrease in the frequency of help suggesting that helper T cells specific for SRBC are perfectly capable of collaborating with a polyclonal population of cytotoxic T precursors in the presence of ConA. The prediction of model 2 is therefore to some extent fulfilled.

The evidence just discussed favors model 2 as the most accurate description of the helper/precursor collaboration induced by ConA. While the evidence is far from conclusive, it is more supportive of the collaborative event occurring as a result of a "nonspecific-glue" and stimulation effect of ConA rather than as an interaction between a precursor cell bearing ConA on its surface and a ConA-specific helper T cell. It is interesting that all attempts to increase the frequency

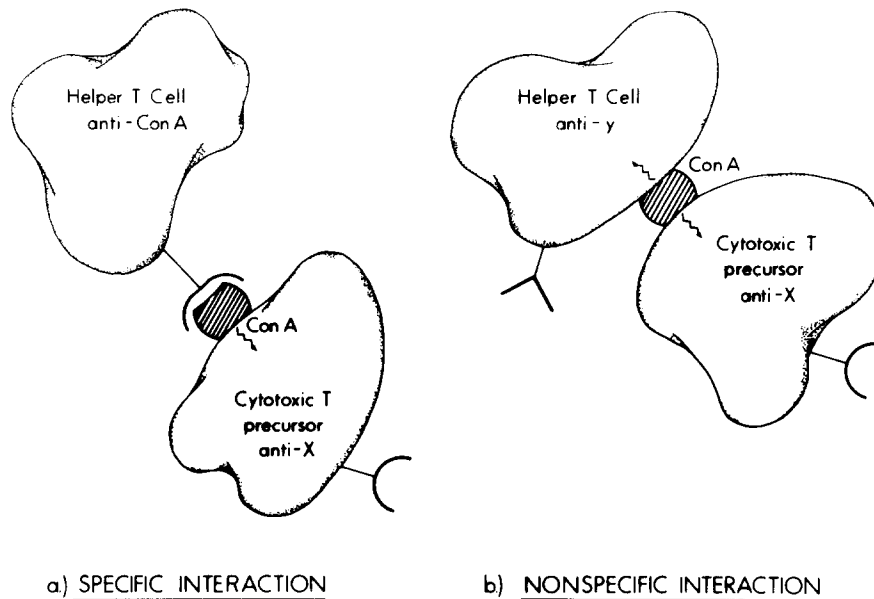


FIG. 3. Possible models for the mechanism by which the ConA-dependent helper effect occurs.

of the helper population were to varying degrees unsuccessful. This suggests that the frequency of "activated" helper T cells in normal spleen or of helper cells which can become activated by ConA stimulation, remains fairly constant, and that specific priming alters the frequency of a specific component of this population without upsetting the overall frequency of helper cell effectors. It is also possible that the ConA-dependent helper effect is considerably less efficient than are antigen-specific interactions. Thus, the frequency of antigen-specific helper cells might appear to increase after priming due to an increased efficiency of antigen-mediated functional interaction rather than an actual numerical increase.

A further interesting aspect of the ConA-dependent generation of cytotoxicity was the enhancement of cytotoxicity observed when the irradiated helper spleen cells were pretreated with ConA. Cytotoxicity was generated in cultures in which the only source of ConA was that on the helper cell surface (Table IX, line 3). Pretreatment of the precursor cell, however, yielded no cytotoxicity in the absence of any further ConA additions (Table IX, line 4). Thus, binding of ConA to the helper cell alone is sufficient to induce a cytotoxic response, and also causes up to sixfold enhancement of cytotoxicity when further ConA is added to the cultures (Table IX, compare line 5 with line 6 and 7). This suggests that the helper cell is a limiting factor in the helper/precursor cell interaction and that pretreatment of the helper cell-containing population optimizes the chances of a successful interaction between the two relevant cells.

Summary

Concanavalin A stimulation of T-cell cytotoxicity has been shown to be absolutely dependent on helper T-cell collaboration. Thymocytes stimulated

with ConA do not differentiate to yield cytotoxic effector cells. However, thymocytes cocultured with irradiated spleen cells as helpers and ConA yield high levels of cytotoxicity. The helper cell bears theta antigens on its surface, is not an adherent cell, and does not require any adherent cell functions in our culture conditions. The ConA-dependent helper cells appear to be polyclonal in specificity. Thus, polyclonal stimulation of cytotoxicity by ConA requires T helper-T precursor collaboration in analogy to antigen-specific T helper-T precursor interactions. Unlike the antigen-specific interactions, the ConA-driven cytotoxicity does not appear to require linked associative recognition for induction of cytotoxicity.

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