HELPER ACTIVITY IS REQUIRED FOR THE IN VIVO GENERATION OF CYTOTOXIC T LYMPHOCYTES*

By JO-ANN KEENE and JAMES FORMAN

From the Department of Microbiology and the Immunology Graduate Program, University of Texas Health Science Center, Dallas, Texas, 75235

The ability of T-helper cells (T_H)[1] to augment the activity of cytotoxic T lymphocytes (CTL) has been extensively characterized (1). In particular, Lyt-1$^+$ T_H or their products (presumably interleukin 2 [IL-2]) have been shown to increase the amount of cytolytic activity generated in in vitro culture systems (2–8). However, it has been difficult to demonstrate that helper activity is requisite for the sensitization of CTL precursors (CTL-P). Thus, while there is an absolute requirement for the addition of helper activity in order to sensitize anti-H-2 and anti-trinitrophenyl (TNP) thymic CTL-P (9, 10), precursors obtained from peripheral lymphoid organs can be sensitized to the same epitopes without the addition of exogenous helper activity (9, 11). This latter observation could reflect either the presence of endogenous T_H in these organs, or the ability of CTL-P to be activated in a helper-cell independent manner. Evidence in favor of the first possibility has been obtained by showing that helper activity is required for the generation of CTL when antigen-presenting cells have been pretreated with physical agents; e.g., heat, ultraviolet light, sonication, etc. (12–14). However, presentation of epitopes on cells treated in the above manner may not accurately reflect the ability of CTL-P to become activated. Therefore, whereas CTL activity can be augmented in peripheral lymphoid organs by T_H, definitive evidence demonstrating a requirement for T_H is lacking.

Even less is known about the role of T_H in allowing for the activation and generation of CTL from CTL-P in vivo. Indirect evidence indicating T_H are needed in vivo was provided by Zinkernagel et al. (15) and von Boehmer and Haas (16), who showed, using bone marrow repopulated radiation chimeras, that there is a requirement for *I* region sharing between CTL and radiation-resistant thymus cells to generate anti-viral and anti-H-Y effector cell activity. Presumably in these instances T_H are *I* region restricted. More direct evidence has been provided by Altman et al. (17) and Butler and Battisto (18), who showed that increased CTL activity is generated against tumor or TNP antigenic determinants if helper antigens are present during sensitization. On the other hand, Bevan (19) showed that in vitro-generated CTL directed against minor-H-antigens required prior in vivo priming but found no evidence to indicate that helper cells were involved.

The data in this report use the CTL response against the Qa-1^b alloantigen. The

[1] *Abbreviations used in this paper:* Con A, concanavalin A; CML, cell-mediated lysis; CTL, cytotoxic T lymphocyte; CTL-P, CTL precursors; IL-2, interleukin 2; *Ir*, immune response; T_H, T helper cells; TNP, trinitrophenyl.

response to this determinant requires prior in vivo priming in order to generate CTL activity in vitro (20-23). The effector cells generated are *H-2* unrestricted and specific for Qa-1, a cell surface glycoprotein that is structurally similar to H-2 class I antigens (24).

The results of our investigation indicate that in vitro CTL-P cannot be generated against the Qa-1 antigen unless animals are first primed in vivo with two different types of antigenic determinants. One of the determinants is Qa-1 and is recognized by CTL-P. However, in the absence of an additional or second (helper) determinant successful in vivo priming does not occur. Recognition of this helper determinant is under *H-2*-linked immune response (*Ir*) gene control. Although we were able to demonstrate an obligatory role for helper determinants in vivo, no such requirement could be detected for the generation of anti-Qa-1 CTL from primed CTL-P in vitro.

Materials and Methods

Animals. A/J mice were obtained from The Jackson Laboratories, Bar Harbor, ME. B6.Tla^a mice were kindly provided by Dr. L. Flaherty, New York State Department of Health, Albany, NY. All other mice were bred in our colonies at the University of Texas Health Science Center at Dallas.

Immunization Procedures. Recipient mice were injected with 40-70 × 10^6 spleen cells intraperitoneally, unless otherwise stated, and sacrificed 3 wk to 4 mo later.

Cytotoxicity Assay. The assay has been previously described (23). Briefly, 5 × 10^6 responder spleen cells and 1 × 10^6 irradiated (3,000 rad) stimulator cells were cultured in 35-mm petri dishes in RPMI 1640 with supplements including 5% fetal calf serum (FCS). Unless otherwise stated, the effector cells were harvested from petri dishes after 5 d in culture, adjusted to the appropriate concentrations in 0.1 ml of medium, and added to individual wells in round-bottomed microtiter plates with 0.1 ml of ^{51}Cr-labeled Isolymph-(Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY) purified concanavalin A (Con A) lymphoblast target cells. These plates were then incubated at 37°C and harvested 4 h later. The data are expressed as net isotope release. This represents the percent ^{51}Cr release from target cells in the presence of in vivo-primed spleen cells cultured with stimulator cells minus the percent ^{51}Cr release from target cells in the presence of in vivo primed spleen cells cultured without stimulator cells. Standard errors of the mean from triplicate samples did not exceed 5%.

Tissue Typing. (A/J × B6.Tla^a)F$_1$ × B6.Tla^a backcross mice were typed for H-2K^k in a microcytotoxicity assay as previously described (25). An anti-H-2K^k IgG$_{2a}$ hybridoma (Salk line 11-4.1) was used together with low-toxicity rabbit complement (Pel-Freez Biologicals, Rogers, AR) to type for the H-2^a haplotype. In each experiment, (A/J × B6.Tla^a)F$_1$ and B6 mice were typed as positive and negative controls, respectively.

Results

B6.Tla^a Mice Do not Respond to the Qa-1^b Alloantigen. There are currently available two *Qa-1/Tla* congenic pairs of mice: (*a*) A.Tla^b, and (*b*) B6.Tla^a, C57BL/6 (B6). A.Tla^b is an A-background mouse that has derived its *Qa-1/Tla* chromosomal segment from B6. Similarly, B6.Tla^a is a B6-background mouse that has derived its *Qa-1/Tla* chromosomal segment from A (Fig. 1). Since *H-2* unrestricted CTL can be generated against the Qa-1^b alloantigen in a secondary cell-mediated lympholysis (CML) assay (20-23), both congenic pairs should be able to generate anti-Qa-1^b CTL. However, we have previously reported (23) that anti-Qa-1^b CTL can be generated in only one of these two congenic pairs. Thus, A/J (*Qa-1^a*) mice can generate CTL when immunized in vivo and restimulated in vitro with Qa-1^b congenic A.Tla^b spleen cells. In contrast, B6.Tla^a (*Qa-1^a*) mice cannot generate CTL when immunized with Qa-1^b congenic B6 spleen cells, using the same protocol.
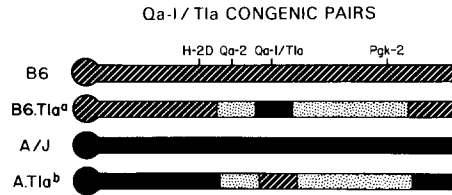
Qa-I / Tla CONGENIC PAIRS

FIG. 1. Schematic representation of the *H-2-Tla* interval on the 17th chromosome of A and B6 Qa/Tla congenic strains. ▨, B6; ■, A/J; ▧, origin of chromosome uncertain, either from B6 or A/J.
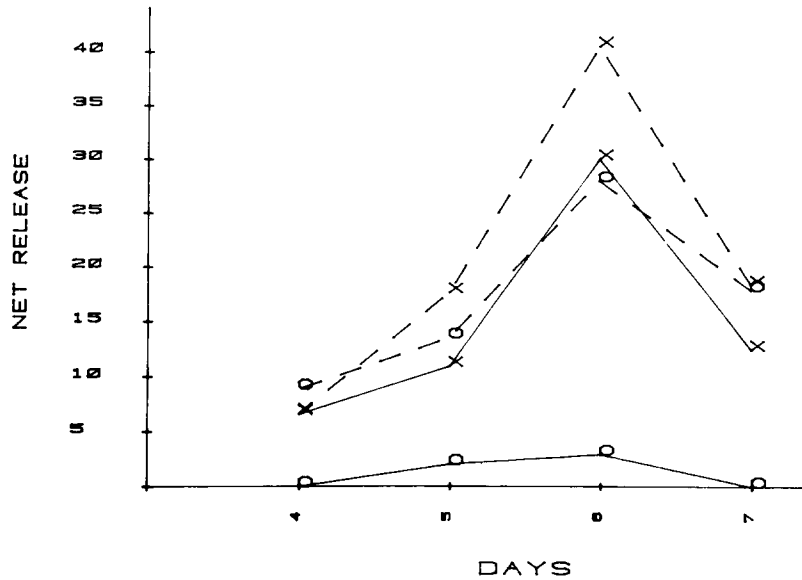

FIG. 2. Kinetics of the anti-Qa-1 response. A/J mice were primed in vivo with Qa-1 congenic spleen cells (A.Tla^b) and restimulated in vitro for a varying number of days (4–7) with either irradiated Qa-1 congenic (A.Tla^b) spleen cells (O----O), or irradiated H-2 allogeneic (B6) spleen cells (X----X). Effector cells were tested for their cytotoxic activity against ^{51}Cr-labeled B6 Con A lymphoblast target cells. B6.Tla^a mice were primed twice in vivo with Qa-1 congenic (B6) spleen cells and restimulated in vitro for a varying number of days with either irradiated Qa-1 congenic (B6) spleen cells (O——O), or irradiated H-2 allogeneic (A/J) spleen cells (X——X). Effector cells were tested against A.Tla^b target cells. Effector/target (E/T) ratio is 100:1.

It is possible that the apparent inability of B6.Tla^a mice to generate anti-Qa-1^b CTL is due to a difference in the kinetics of the response in B6.Tla^a mice relative to A strain mice. Accordingly, both A/J and B6.Tla^a mice were primed in vivo with Qa-1 congenic spleen cells and restimulated in vitro for a varying number of days with either irradiated Qa-1 congenic spleen cells or irradiated H-2 allogeneic spleen cells as a control (Fig. 2). In all experiments where anti-Qa-1 CTL activity was tested, A/J anti-A.Tla^b effector cells were tested against B6 targets, whereas B6.Tla^a anti-B6 effectors were tested against A.Tla^b targets. This was done to insure that we were measuring *H-2*-unrestricted anti-Qa-1^b CTL activity. A/J mice generated both anti-Qa-1^b and anti-H-2 cytotoxic activity with peak lytic values noted at 6 d. In contrast, although B6.Tla^a strain mice generated anti-H-2 CTL, at no time did they show any

anti-Qa-1^b cytotoxic activity. Therefore, B6.Tla^a mice are nonresponders throughout the time course of the in vitro assay.

Although intraperitoneal inoculation did not sensitize B6.Tla^a mice to Qa-1^b on B6 splenocytes, other routes of immunization might be more effective. Accordingly, various routes of in vivo immunization including intraperitoneal, intravenous, subcutaneous, and via the footpad were tested. The data presented in Fig. 3 demonstrate that the ability of A/J or the inability of B6.Tla^a mice to respond was not altered by any of these protocols. Further, whereas a single inoculation of 30 × 10^6 B6 spleen cells in vivo is ineffective in sensitizing B6.Tla^a mice, multiple injections or an increased number of cells in the inoculum might allow for the generation of anti-Qa-1^b CTL. However, neither two inoculations of priming cells (Fig. 1) nor increasing the number of cells in the inoculum to 115 × 10^6 (data not shown) resulted in anti-Qa-1 CTL.

The Inability of B6.Tla^a Mice to Respond to Qa-1^b Is not due to an Absence of CTL-P.   The inability of B6.Tla^a mice to generate anti-Qa-1^b CTL could be due to either an absence of anti-Qa-1^b CTL-P or an inability to sensitize CTL-P in these animals. Evidence in favor of the latter possibility was obtained by in vivo priming B6.Tla^a mice with spleen cells from animals other than B6. Thus, B6.Tla^a mice do generate anti-Qa-1^b CTL when immunized in vivo with A.BY rather than B6 spleen cells (Fig. 4). A.BY mice have the same *H-2* and *Qa-1* alleles as B6 (*H-2^b*, *Qa-1^b*) but differ from B6 (and B6.Tla^a) at multiple background loci.

In addition to A.BY cells, female B6.Tla^a nonresponder mice also generate anti-Qa-1^b CTL when immunized in vivo with B6 male spleen cells (Fig. 4). Apparently,
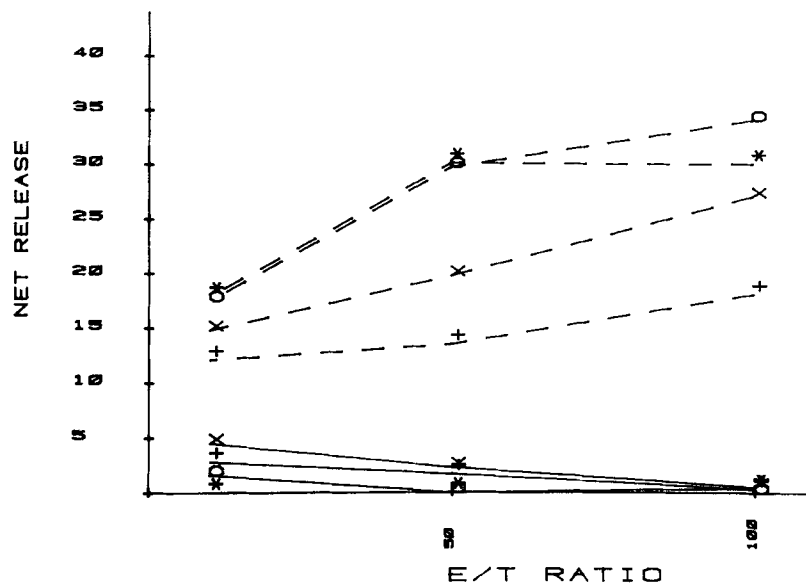

Fig. 3.   The route of immunization does not alter the ability to respond to Qa-1. A/J (– – –) and B6.Tla^a (———) mice were primed in vivo with Qa-1 congenic spleen cells (A.Tla^b and B6, respectively) by various routes of immunization (intraperitoneal (•), intravenous (○), subcutaneous (×), and via the footpad (+), and restimulated in vitro with irradiated Qa-1 congenic spleen cells. A/J and B6.Tla^a effector cells were tested for their cytotoxic activity against ^{51}Cr-labeled Con A lymphoblast Qa-1^b target cells (B6 and A.Tla^b, respectively) at E/T ratios of 10:1, 50:1, and 100:1.
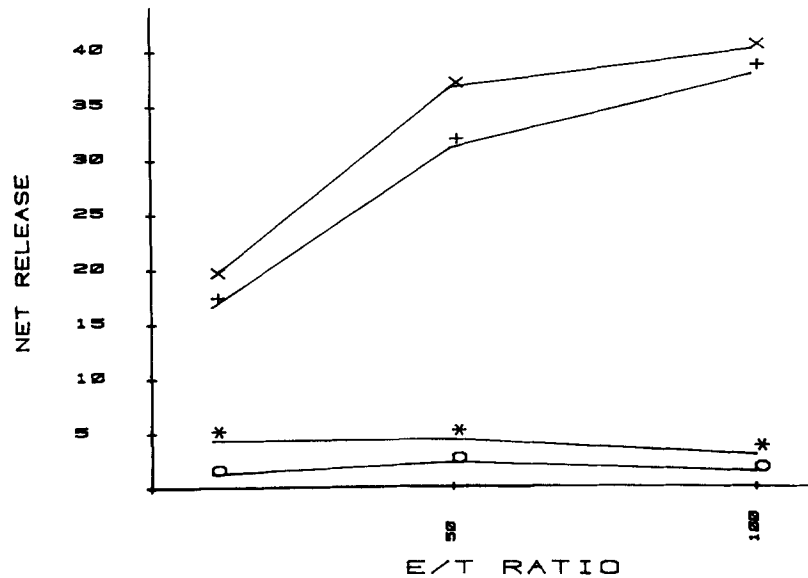
Fig. 4. B6.Tla^a mice have CTL-P that recognize Qa-1^b. B6.Tla^a male mice were primed in vivo with sex-matched B6 spleen cells (O) or A.BY spleen cells (+) and restimulated in vitro with sex-matched Qa-1 congenic B6 splenocytes. B6.Tla^a female mice were primed in vivo with sex-matched B6 spleen cells (*) or B6 male cells (X) and restimulated in vitro with B6 male splenocytes. Effector cells were tested for cytotoxic activity against ^51Cr-labeled Con A lymphoblast A.Tla^b target cells from female mice at E/T ratios of 10:1, 50:1, and 100:1.

the addition of the H-Y antigenic disparity on the immunizing cells converts female B6.Tla^a nonresponder mice into responders. It is important to note that the effector cells generated are specific for the Qa-1^b alloantigen and not H-Y, because the target cells used in the assay are obtained from female mice. Therefore, the above data demonstrate that the inability of B6.Tla^a mice to respond to the Qa-1^b alloantigen when primed with B6 spleen cells is not due to a lack of CTL-P, but rather the inability of B6 cells to sensitize CTL-P in vivo.

The Requirement for H-Y and Qa-1 To Be Presented on the Same Immunizing Cell. The ability of B6.Tla^a mice to respond to Qa-1 if primed with non-Qa-1 congenic spleen cells could be due to a nonspecific adjuvant effect induced by the extra antigen. On the other hand, in analogy with T-B collaboration, there may be a requirement for linked recognition suggesting specific recognition of the extra determinant. This issue was addressed by determining if both antigens, Qa-1 and H-Y, need to be on the same cell used for in vivo priming.

B6.Tla^a female mice were immunized intraperitoneally with a mixture of 30×10^6 B6.Tla^a male spleen cells and 30×10^6 B6 female spleen cells. Accordingly, each cell presents either the H-Y or the Qa-1 alloantigen, respectively. The data presented in Table I demonstrate a requirement for both antigenic determinants to be presented on the same cell for successful immunization. Accordingly, cytotoxic activity is generated when B6.Tla^a female mice are primed in vivo with B6 male spleen cells but not B6 female cells, as expected (lines 1 and 2). However, no cytotoxic activity was generated when H-Y and Qa-1 were presented on separate cells (lines 3 and 4), even when both antigens were presented on the same cell (line 3) during in vitro restimu-

TABLE I

H-Y and Qa-1 Must Be Present on the Same Cell for Successful Priming Intraperitoneally

Line	Responder cells	Stimulator cells		Target cells	Net ^{51}Cr release		
		In Vivo	In Vitro		100	50	10
1	B6.Tla^a ♀	B6 ♀	B6 ♀	A.Tla^b ♀	1.2	0.9	0.5
2		B6 ♂	B6 ♂		53.2	49.9	34.4
3		B6.Tla^a ♂ + B6 ♀	B6 ♂		0.9	0.9	1.5
4		B6.Tla^a ♂ + B6 ♀	B6.Tla^a ♂ + B6 ♀		5.3	7.4	4.8

B6.Tla^a female mice were immunized intraperitoneally with 30 × 10^6 B6 female spleen cells, 30 × 10^6 B6 male spleen cells, or a mixture of 30 × 10^6 B6.Tla^a male spleen cells and 30 × 10^6 B6 female spleen cells. Effector cells were restimulated in vitro and tested for cytotoxic activity on ^{51}Cr-labeled Con A lymphoblast A.Tla^b female target cells. Effector cells were also tested against A/J female targets. Net ^{51}Cr release did not exceed 15.3% at 100:1 effector/target ratio.

TABLE II

Requirement for H-Y and Qa-1 To be Present on the Same Cell for Successful Priming via the Footpad

Responder cells	Stimulator cells		Target cells	Lytic* units
	In Vivo	In Vitro		
B6.Tla^a ♀	B6 ♂	B6 ♂	A.Tla^b ♀	100
	B6.Tla^a ♂ + B6 ♀			5

B6.Tla^a female mice were immunized via the footpad with 30 × 10^6 B6 male spleen cells or a mixture of 30 × 10^6 B6.Tla^a male and 30 × 10^6 B6 female spleen cells. Effector cells were restimulated in vitro and tested for cytotoxic activity on ^{51}Cr-labeled Con A lymphoblast A.Tla^b female target cells.
* Lytic units = (Number input responder cells/culture × 10^6 required for 25% net release)$^{-1}$ × 10^3.

lation. Thus, only when B6.Tla^a female mice are immunized with B6 male spleen cells (H-Y and Qa-1 presented on the same cell) is an anti-Qa-1^b CTL response elicited. In this manner, H-Y is analogous to a carrier determinant requiring linked recognition, rather than acting as an adjuvant.

Due to the limited size of murine footpads and the restricted lymphatic drainage of the footpad as compared to the peritoneal cavity, the requirement for linked recognition might be circumvented if mice were immunized via the footpad. Therefore, B6.Tla^a female mice were immunized via the footpad with either 30 × 10^6 B6 male cells or a mixture of 30 × 10^6 B6.Tla^a male cells and 30 × 10^6 B6 female cells (Table II). Similar to previous results (see Table I), significant anti-Qa-1 cytotoxic activity was generated after in vivo priming with B6 male cells (Table II). However, when the H-Y and Qa-1 antigens were presented on separate cells, an anti-Qa-1 response was observed in only two out of four experiments. In one of the two positive experiments, presented in Table II, the number of lytic units generated was only 5% of that obtained when both antigens were presented on the same cell. Similar results were noted in the other experiment. Therefore, regardless of the route of immunization, both antigens must be presented on the same cell for successful sensitization.

The Carrier Determinant Is only Required for In Vivo Priming. Because the CTL response can only be elicited after both in vivo priming and in vitro challenge, the requirement for the presence of a carrier determinant for each of these two phases of the response

was analyzed. The data in Table III demonstrate that B6.Tla^a female mice only respond to Qa-1^b if primed in vivo with B6 male cells, as expected (line 1 vs. 3). Further, when the helper (H-Y) antigen is present in vitro but not in vivo no cytotoxic activity is generated (line 2). However, if B6.Tla^a female mice are primed in vivo with B6 male cells, they can then be successfully restimulated in vitro with Qa-1 disparate cells possessing H-Y determinants (line 4) or lacking H-Y determinants (line 3). Therefore, the extra determinant(s) is only required for in vivo priming and is not needed for in vitro restimulation.

Even though the carrier determinant is not required during the in vitro phase of the response, it is possible that it could augment the CTL activity generated by activating T$_H$ in vitro. Therefore, we compared the amount of CTL activity generated in vitro in the presence or absence of the helper (H-Y) determinant. The data in Table IV, together with the data in Table III, show that an equivalent amount of CTL activity is generated following in vitro restimulation with either B6 female or male cells.

Priming with the Helper Antigen Only Does not Allow for the Generation of CTL-P In Vitro. The previous data suggest that the addition of extra antigenic (helper) determinants during in vivo priming allows for the generation of CTL-P, which can be driven to CTL in vitro. However, it is possible that the helper determinants act by increasing the number of T$_H$ cells so that existing unprimed CTL-P can be activated in vitro. According to this alternative, CTL.P per se do not require in vivo priming.

TABLE III
H-Y (Helper) Determinant Is Required Only during In Vivo Priming

Line	Responder cells	Stimulator cells		Target cells	Net ^{51}Cr release		
		In Vivo	In Vitro		100	50	10
1	B6.Tla^a ♀	B6 ♀	B6 ♀	A.Tla^b ♀	0.2	0.0	0.5
2			B6 ♂		0.2	0.2	−0.1
3		B6 ♂	B6 ♀		41.5	39.1	18.2
4			B6 ♂		45.8	40.0	23.1

B6.Tla^a female mice were primed in vivo and restimulated in vitro with either B6 female spleen cells or B6 male spleen cells. Cytotoxic activity was tested on ^{51}Cr-labeled Con A lymphoblast A.Tla^b female target cells.

TABLE IV
There Is no Carrier Augmentation In Vitro

Line	Responder cells	Stimulator cells		Target cells	Net ^{51}Cr release		
		In Vivo	In Vitro		100	50	10
1	B6.Tla^a ♀	B6 ♂	B6 ♀	A.Tla^b ♀	38.4	36.8	23.3
2			B6 ♂		37.9	34.0	28.3
3			B6 ♀	B10.S	54.4	44.2	24.3
4			B6 ♂		39.4	34.4	15.6

B6.Tla^a female mice were primed in vivo with B6 male spleen cells and restimulated in vitro with the above panel of spleen cells. Effector cells were tested against ^{51}Cr-labeled Con A lymphoblast A.Tla^b female or B10.S (Qa-1^b) target cells.

To investigate these possibilities, B6.Tla^a female mice were primed in vivo with cells containing the helper (H-Y) determinant only (B6.Tla^a male cells) and restimulated in vitro with either Qa-1^b and H-Y$^-$ (B6 female) cells or Qa-1^b and H-Y$^+$ (B6 male) cells. Controls included in vitro restimulation with H-Y$^+$ (B6.Tla^a male) cells and testing for anti-H-Y cytolytic activity.

The data presented in Table V show that no anti-Qa-1 cytolytic activity was generated when B6.Tla^a female mice were primed in vivo with B6.Tla^a male cells and rechallenged with stimulator cells in vitro that express both helper and Qa-1 determinants (line 2). As a positive control, we show that B6.Tla^a female mice do respond when primed in vivo with cells expressing both H-Y and Qa-1 (line 3). Whereas inoculation with B6.Tla^a male cells did not prime for anti-Qa-1 CTL, it did prime for anti-H-Y CTL (lines 5 and 6). Thus, in vivo priming with only helper determinants does not allow for an in vitro anti-Qa-1 response although helper determinant responsive cells were present in the in vitro culture.

The Inability of B6.Tla^a Mice to Respond Is Controlled by an Immune Response Gene. The ability of A/J animals to respond to A.Tla^b, whereas B6.Tla^a mice do not respond to B6, could be due to possible additional antigenic differences at *Qa-1*-linked loci that exist between the A strain congenics but are not present between the B6 strain congenics. These additional alloantigenic differences could include helper antigens similar to H-Y. Alternatively, A/J mice may possess an *Ir* gene(s) that permits the recognition of helper determinants on the Qa-1 molecule itself. This issue was addressed by immunizing (A/J × B6.Tla^a)F$_1$ mice with sex-matched B6 spleen cells. Because B6 stimulator cells possess no additional antigenic determinants that can function to stimulate helper cell activity, these F$_1$ animals should not be able to respond to Qa-1^b unless there is an A strain *Ir* gene. As shown in Fig. 5, (A/J × B6.Tla^a)F$_1$ animals do respond to Qa-1^b on sex-matched B6 stimulator cells. Thus, the ability to respond to the Qa-1^b alloantigen in the absence of an added helper determinant is encoded for by a dominant gene(s) in A/J mice.

To determine whether the *Ir* gene(s) involved in the recognition of helper determinants is linked to the *H-2* complex, (responder × nonresponder)F$_1$ mice were backcrossed to the nonresponding parent (B6.Tla^a) and the progeny were tissue typed and tested for anti-Qa-1^b CTL activity (Table VI). Of the 18 backcross animals tested, we found that 10 animals were able to generate an anti-Qa-1^b CTL response

TABLE V

Carrier Priming Does Not Allow for a Primary In Vitro Response

Line	Responder cells	Stimulator cells		Target cells	Net ^{51}Cr release		
		In Vivo	In Vitro		100	50	10
1	B6.Tla^a ♀	B6.Tla^a ♂	B6 ♀	A.Tla^b ♀	11.8	8.8	4.7
2			B6 ♂		11.0	10.3	3.6
3		B6 ♂	B6 ♀		38.4	36.8	23.3
4		B6.Tla^a ♂	B6 ♀	B6.Tla^a ♂	−2.3	−3.4	−2.3
5			B6 ♂		37.6	31.7	21.6
6			B6.Tla^a ♂		37.0	33.8	17.9

B6.Tla^a female mice were primed in vivo with B6.Tla^a male spleen cells and restimulated in vitro with the above panel of spleen cells. Effector cells were tested against both ^{51}Cr-labeled Con A lymphoblast A.Tla^b female and B6.Tla^a male target cells.
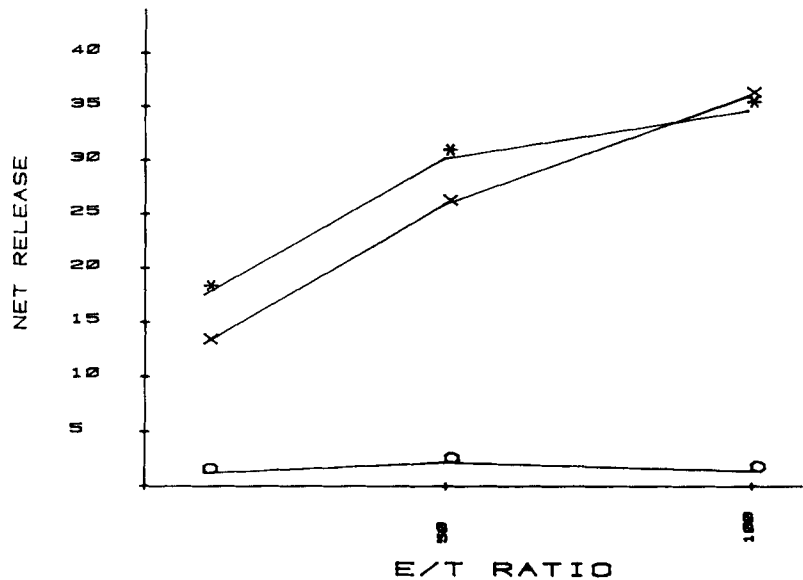
Fig. 5.   The ability of (A/J × B6.Tla^a)F$_1$ mice to respond. (A/J × B6.Tla^a)F$_1$ mice (∗) and B6.Tla^a mice (○) were primed in vivo and restimulated in vitro with sex-matched B6 spleen cells. Cytotoxic activity was tested against ^{51}Cr-labeled Con A lymphoblast A.Tla^b target cells. A/J mice (×) were primed in vivo and restimulated in vitro with sex-matched A.Tla^b spleen cells and were tested against B6 target cells. E/T ratios shown are 10:1, 50:1, and 100:1.

TABLE VI

Ability of Backcross Animals to Generate an Anti-Qa-1^b Response *

	H-2 haplotype§		
Anti-Qa-1^b response‡	H-2^a/H-2^b	H-2^b/H-2^b	Totals
Positive	9	1	10
Negative	2	6	8
Totals	11	7	

* (A/J × B6.Tla^a)F$_1$ mice were backcrossed to the nonresponding parent (B6.Tla^a). These (A/J × B6.Tla^a)F$_1$ × B6.Tla^a mice were primed in vivo and restimulated in vitro with sex matched B6 spleen cells. Cytotoxic activity was tested on ^{51}Cr-labeled Con A lymphoblast A.Tla^b target cells.
‡ Positive anti-Qa-1^b responses represent a net ^{51}Cr release of >10% at effector/ target of 100.
§ Tissue typing was performed as described in Materials and Methods.

when primed in vivo with sex-matched B6 cells, whereas 8 animals were not. To determine whether the A/J Ir gene is H-2 linked, these same animals were tissue typed for the responding H-2^a (H-$2K^kIA^kD^d$) haplotype using an anti-H-2K^k monoclonal antibody. 9 of 11 H-2^a/H-2^b heterozygotes responded, while 6 of 7 H-2^b/H-2^b homozygotes did not respond, data consistent with the Ir gene being H-2 linked (P < 0.035). Because 2 of 11 H-2^a/H-2^b animals did not respond, an additional non-H-2 linked Ir gene may also be involved. The ability of one of the seven H-2^b/H-2^b homozygotes to respond may be explained by recombination between H-$2K^b$ (the tissue typing reagent was anti-H-2K^k) and IA^k.

Discussion

The data in this report demonstrate that peripheral CTL-P directed against the alloantigen Qa-1^b require in vivo priming with two kinds of antigenic determinants to generate a secondary CTL response in vitro. One of the determinants is found on the Qa-1 molecule itself and is the target antigen for the CTL effector cells. The second determinant need not be on Qa-1, is not recognized by anti-Qa-1 CTL, but must be presented together with Qa-1 to prime CTL-P in vivo. We have termed this second specificity a helper determinant since this defines its functional role in the generation of CTL effector cells. Although this helper determinant is required in vivo, its presence it not requisite for the secondary in vitro boosting of primed CTL-P. Further, recognition of the helper determinant is under H-2-linked Ir gene control.

Our ability to demonstrate the role of helper determinants in allowing for CTL-P priming in vivo was afforded by the observation that B6.Tla^a (Qa-1^a) mice confronted with congenic spleen cells from B6 (Qa-1^b) donors failed to mount an anti-Qa-1^b CTL response. The inability of B6.Tla^a animals to respond was not due to a lack of CTL-P against the Qa-1^b alloantigen, because in vivo priming with noncongenic Qa-1 disparate splenocytes from A.BY mice successfully primed these animals for subsequent in vitro anti-Qa-1 CTL activity. In addition to multiple minor-H-antigens, defined by the difference in background genes between A and B6 animals, we also demonstrated that the H-Y antigen can act as a helper determinant as B6.Tla^a (Qa-1^a) female mice could be successfully primed in vivo with B6 (Qa-1^b) spleen cells from male animals. Therefore, extra antigens on the noncongenic cells act as helper determinants and allowed us to dissect the role of both CTL and helper determinants that allow for an anti-Qa-1 CTL response.

There are several reports indicating that the addition of extra antigens augments both CTL and humoral responses against cell membrane alloantigens. For example, Lake and Douglas (26) have shown that anti-Thy-1 humoral responses are augmented when the donor-recipient pairs are noncongenic for the Thy-1 gene. Wernet and Lilly (27) found that anti-H-2D^b alloantisera of the IgG class could not be raised by cross immunizing mice congenic at the D-end of the H-2 complex, whereas this sera could be produced using noncongenic recipients. Butler and Battisto (18) showed that the primary in vivo anti-TNP CTL response of popliteal lymph node cells following a footpad inoculation of TNP-derivatized cells could only occur if the TNP cells came from Mls disparate animals.

The role of a helper antigen could be thought of in two ways; (a) as an adjuvant that nonspecifically augments an immune response, or (b) as a carrier determinant as defined in the linked-recognition interpretation of T-B collaboration (28). We have addressed this issue by priming animals with the CTL and helper determinants presented on separate antigen-presenting cells. Our data clearly show that both the CTL (Qa-1^b) and the helper (H-Y) determinants need be on the same cell for in vivo priming, thus supporting the proposition that linked recognition involving an interaction between cells recognizing CTL and helper determinants is required. This was not only observed using the intraperitoneal route of inoculation, but also when cells were injected into the same footpad, and differs from the results of Butler and Battisto (18) who found that TNP and Mls determinants could be presented on different cells.

Zinkernagel et al. (15) and von Boehmer and Haas (16), using bone marrow-reconstituted radiation chimeras, showed a necessity for I region sharing between

donor lymphocytes and host radio-resistant thymus cells to generate antigen-specific cytotoxic T cell activity. However, even if it is assumed that the requirement for I region compatibility reflects the ability to sensitize helper cells, this approach does not allow for a determination of when helper cell activity is required. More direct evidence has come from the experiments of Altman et al. (17) and Hamaoka et al. (29) who showed that primed anti-TNP T_H augment the in vitro CTL response against a syngeneic tumor. However, in these experiments anti-tumor responses could be induced without the addition of the TNP (helper) determinant. Further, animals confronted with TNP-tumor cells had to be pretreated with dinitrophenol-GL before priming for helper cell activity, otherwise suppression rather than augmentation of the CTL response occurred. Therefore, it is not clear whether the anti-TNP helper cells were required for generating CTL-P in vitro or for overcoming the activity of TNP-specific suppressor cells. Green et al. (30) have shown that in vitro CTL activity against EδG2 cells, a B6 leukemia bearing AKR/Gross virus antigens, requires in vivo priming with AKR leukemia cells to generate in vitro CTL-P that can be sensitized against EδG2 cells. Although a variable expression of tumor antigens or a difference in immunogenicity of these leukemia lines may in part account for their results, it is likely that the H-2^k alloantigens serve as helper determinants, similar to H-Y in the anti-Qa-1 response.

Recognition of the helper determinant in vivo may be required either to generate in vitro CTL-P, or alternatively, prime T_H, which subsequently activate unprimed CTL-P in vitro. We used two approaches to distinguish between these possibilities. First, we showed that animals primed in vivo only with the H-Y antigen could not generate anti-Qa-1 CTL activity when challenged in vitro with stimulator cells containing both Qa-1 and H-Y. Second, omission of the helper antigen by using female stimulator cells in vitro still allowed for the generation of anti-Qa-1^b CTL activity. Taken together, our data demonstrate that helper determinants must be presented during in vivo priming together with CTL determinans in order to prime peripheral CTL-P.

Whereas we were able to show that helper determinants are requisite during in vivo priming of anti-Qa-1 CTL-P, no such requirement could be demonstrated in vitro. Accordingly, cultures of B6.Tla^a female splenocytes taken from animals primed with B6 male cells generated as much cytolytic activity when challenged in vitro with B6 female as B6 male stimulator cells. While this demonstrates that the helper determinant is not required for the in vitro phase of the CTL response, it does not rule out a possible contribution of putative helper activity, because anti-Qa-1^b CTL-P in vitro must undergo cell division (unpublished results). Thus, it is possible that only a small amount of helper activity is required for the in vitro CTL response and can be provided by recognition of the Qa-1^b antigen, itself, whereas during in vivo priming more helper activity is required and can only be provided by recognition of the additional (H-Y) helper determinant.

Other laboratories have been unable to demonstrate an obligatory role for T_H, as defined by the addition of IL-2, in the generation of peripheral CTL in vitro. Thus, with the exception of thymocytes (9, 10), peripheral CTL-P can be sensitized without the addition of exogenous IL-2. The ability to sensitize CTL-P without the addition of IL-2 could either reflect the presence of IL-2-secreting T_H in peripheral lymphoid organs or the fact that CTL-P do not require this helper (IL-2) signal. If the latter

case is correct, then we suggest that the helper signal has been previously supplied in vivo. This implies that all CTL responses measured in vitro are derived from primed CTL-P, and is consistent with the data of Finberg et al. (31), who have postulated that anti-H-2 CTL-P are derived from cells previously exposed to environmental antigens.

Several investigators (12–14) have suboptimized in vitro culture conditions by treating stimulator cells with physical agents such as ultraviolet light, heat, sonication, etc., to show that peripheral CTL-P require the addition of helper signals or IL-2 to generate CTL-effector activity. However, these treatments may not reflect the ability of CTL-P to become activated when confronted with antigen on untreated stimulator cells. Finberg et al. (11) and Fujiwara and Shearer (32) have demonstrated that helper activity can augment anti-TNP CTL responses in vitro. Interestingly, Finberg et al. (11) have also shown that anti-TNP suppressor cells can inhibit the augmentation of anti-TNP CTL activity observed in the presence of T_H, but cannot inhibit the residual CTL activity seen in the absence of exogenous T_H. Those data, together with the evidence provided in this report, suggests that T_H activity may not be required for the in vitro generation of CTL from CTL-P. If CTL are generated in the absence of IL-2, we suggest that there is a relatively high frequency of antigen-specific CTL-P, and that at least a few rounds of cell division can occur in the absence of IL-2, which would be sufficient to produce detectable CTL activity.

Ashman and Mullbacher (33) have shown that primed helper cells added to in vitro cultures of unprimed spleen cells can allow for a primary anti-viral CTL response. Thus, under some circumstances helper activity can be demonstrated as necessary in vitro. Since it would be expected that unprimed mice would have only a small frequency of specific anti-viral CTL-P, several rounds of cell division may be required to amplify clones to produce detectable effector activity, thus making the response more IL-2 dependent.

The ability to recognize the helper determinant on the Qa-1 antigen is under H-2-linked Ir-gene control. This was demonstrated by the finding that all (B6.Tla^a × A/J)F$_1$ mice generated anti-Qa-1 CTL when confronted with Qa-1^b on B6 splenocytes and that 9 of 10 responding backcross animals were H-$2^a/H$-2^b heterozygotes. Therefore, these results are in agreement with other data investigating the role of Ir genes
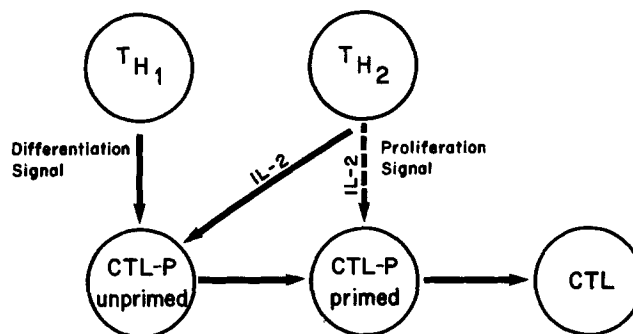

Fig. 6. A model for minimal cellular interactions required in the generation of CTL. Unprimed CTL-P in vivo require a differentiation signal from one helper cell (T_{H1}) and a proliferation (IL-2) signal from a second helper cell (T_{H2}) in the presence of antigen to generate CTL effector and memory (primed CTL-P) cells. In vitro restimulation (---) of primed CTL-P requires only a proliferation signal in the presence of antigen.

in CTL recognition of antigen (16). Since 2 of 11 $H\text{-}2^a/H\text{-}2^b$ heterozygotes were nonresponders to Qa-1, it is also possible that a second non-H-2-linked Ir gene may be involved.

While the data in this report can be interpreted as indicating that helper determinants activate IL-2 secreting Th in vivo to allow for sensitization of CTL-P, we would like to propose an alternative hypothesis diagramed in Fig. 6. This hypothesis is based partly on the findings that the expansion and differentiation of B cell precursors into antibody-secreting cells requires at least two different T cell activities (34–36), and the proposition that IL-2 is requisite during in vitro culture to generate CTL from CTL-P. Accordingly, we propose that the H-Y helper determinant activates a helper cell (T$_{H1}$) that provides a differentiation signal to CTL-P that must be delivered in the presence of antigen (Qa-1). Following or together with that interaction, a second signal, IL-2, elaborated by a different helper cell (T$_{H2}$), allows for proliferation of CTL-P into both CTL effector and memory cells, the latter being indistinguishable from primed CTL-P. During in vitro culture, primed CTL-P only require an IL-2 signal from one of these helper cells, T$_{H2}$, to generate effector cells. According to our hypothesis, it follows that the defect in B6.Tla^a animals is not a lack of IL-2 secreting T$_{H2}$, but rather in T$_H$1 that provide a differentiation signal. Therefore, a testable prediction of the model is that B6.Tla^a animals have anti-Qa-1 T$_{H2}$. Experiments are currently in progress to test this possibility.

Summary

B6.Tla^a ($Qa\text{-}I^a$) mice that are primed in vivo and restimulated in vitro with Qa-1 congenic spleen cells from B6 ($Qa\text{-}I^b$) animals are unable to generate anti-Qa-1^b cytotoxic T lymphocytes (CTL). This nonresponsive pattern was observed regardless of the route of immunization or the time of testing in vitro.

Although B6.Tla^a mice are nonresponders to Qa-1^b when presented on B6 cells, these mice can generate anti-Qa-1^b CTL when primed in vivo with Qa-1 and H-Y alloantigens (females primed with B6 male cells) or Qa-1 and minor-H- alloantigens (primed with sex-matched A.BY cells). Therefore, the inability to generate anti-Qa-1^b CTL is due to a lack of helper or accessory antigens on B6 immunizing cells obligatory during in vivo priming, rather than an absence of anti-Qa-1^b CTL precursors (CTL-P). Demonstration that the additional determinants required during in vivo priming actually function as carrier or helper determinants was shown by the requirement for linked recognition of Qa-1 and the helper determinants (H-Y) in vivo, and the fact that H-Y was not present on susceptible target cells. Animals primed in vivo with H-Y only could not generate anti-Qa-1 CTL activity when challenged in vitro with both Qa-1 and H-Y, indicating that recognition of the helper determinant causes in vivo priming of CTL-P rather than generating helper activity that might activate unprimed CTL-P in vitro.

Whereas unprimed peripheral CTL-P require the presence of both Qa-1 (CTL) and H-Y (helper) determinants for successful in vivo priming, helper determinants were not required in vitro because primed CTL-P from B6.Tla^a mice could be driven to CTL in vitro using sex-matched B6 stimulator cells.

The generation of anti-Qa-1^b CTL is under immune response (Ir) gene control because F$_1$ mice, obtained by crossing responder A/J with nonresponder B6.Tla^a animals, generated CTL to the Qa-1^b alloantigen when presented on B6 spleen cells.

Progeny testing of backcross mice further demonstrated that the *Ir* gene(s) is linked to the *H-2* complex.

These data indicate that an *H-2*-linked *Ir* gene controls the recognition of helper determinants required for CTL priming in vivo. These helper determinants can be distinguished from CTL determinants and both must be recognized together for successful priming of CTL-P.

We wish to thank Bettye Jo Washington for her typing of this manuscript.

References

1. Moller, G., editor. 1980. T cell stimulating growth factors. *Immunol. Rev.* 51:1.
2. Altman, A., and I. R. Cohen. 1975. Cell-free media of mixed lymphocyte cultures augmenting sensitization *in vitro* of mouse T lymphocytes against allogeneic fibroblasts. *Eur. J. Immunol.* 5:437.
3. Plate, J. 1976. Soluble factors substitute for T-T-cell collaboration in generation of T-killer lymphocytes. *Nature (Lond.).* 260:329.
4. Finke, J. M., C. G. Orosz, and J. R. Battisto. 1977. Splenic T-killer cells can be generated by allogeneic thymic cells in conjunction with assisting factor. *Nature (Lond.).* 267:353.
5. Wagner, H., M. Rollinghoff, K. Pfizenmaier, C. Hardt, and G. Johnscher. 1980. T-T cell interactions during in vitro cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt-1⁺ T cells is rate limiting i) in T cell responses to nonimmunogenic alloantigen, ii) in thymocyte responses to allogeneic stimulator cells, and iii) recruits allo- of H-2 restricted CTL precursors from the Lyt-123⁺ T subset. *J. Immunol.* 124:1058.
6. Simon, P. L., J. J. Farrar, and P. D. Kind. 1977. The xenogeneic effect. III. Induction of cell-mediated cytotoxicity by alloantigen-stimulated thymocytes in the presence of xenogeneic reconstitution factor. *J. Immunol.* 118:1129.
7. Rulon, I., and D. W. Talmage. 1979. Enhancement of the primary cytotoxic response to membrane by a lymphokine costimulator. *Proc. Natl. Acad. Sci. U. S. A.* 76:1994.
8. Lalande, M. E., M. J. McCutcheon, and R. G. Miller. 1980. Quantitative studies on the precursors for cytotoxic lymphocytes. VI. Second signal requirements of specifically activated precursors isolated 12 h after stimulation. *J. Exp. Med.* 151:12.
9. Pilarski, L. M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. *J. Exp. Med.* 145:709.
10. Cooley, M. A., and A. Schmitt-Verhulst. 1979. Specific helper T cells permit differentiation of thymic anti-self-trinitrophenyl cytotoxic precursor cells. *J. Immunol.* 123:2328.
11. Finberg, R., S. J. Burakoff, B. Benacerraf, and M. I. Greene. 1979. The cytolytic T lymphocyte response to trinitrophenyl-modified syngeneic cells. II. Evidence for antigen-specific suppressor T cells. *J. Immunol.* 123:1210.
12. Okada, M., G. R. Klimpel, R. C. Kuppers, and C. S. Henney. 1979. The differentiation of cytotoxic T cells *in vitro*. I. Amplifying factor(s) in the primary response is Lyt-1⁺ cell dependent. *J. Immunol.* 122:2527.
13. Pilarski, L. M. 1979. Antigen-specific helper T cells are essential for cytotoxic T cell responses to metabolically inactivated stimulator cells. *Eur. J. Immunol.* 9:454.
14. Bach, F. H., C. Grillot-Courvalin, O. J. Kuperman, H. W. Sollinger, C. Hayes, P. M. Sondel, B. J. Alter, and M. L. Bach. 1977. Antigenic requirements for triggering of cytotoxic T lymphocytes. *Immunol. Rev.* 35:76.
15. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. *J. Exp. Med.* 147:897.

16. von Boehmer, H., and W. Haas. 1979. Distinct *Ir* genes for helper and killer cells in the cytotoxic response to H-Y antigen. *J. Exp. Med.* 150:1134.

17. Altman, A., T. E. Bechtold, and D. H. Katz. 1979. Manipulation of cytotoxic T lymphocyte responses to syngeneic tumors. I. Hapten-reactive helper T lymphocytes augment tumor-specific CTL responses in vitro. *J. Immunol.* 122:2428.

18. Butler, L. D., and J. R. Battisto. 1979. *In vivo* generation of hapten-specific killer T cells without elimination of suppressor cells. *J. Immunol.* 118:1370.

19. Bevan, M. 1977. Priming for a cytotoxic response to minor histocompatibility antigens: antigen specificity and failure to demonstrate a carrier effect. *J. Immunol.* 118:1370.

20. Klein, J., and C. Chiang. 1978. A new locus (H-2T) at the D end of the H-2 complex. *Immunogenetics.* 6:235.

21. Kastner, D. L., and R. R. Rich. 1979. H-2-nonrestricted cytotoxic response to an antigen encoded telomeric to H-2D. *J. Immunol.* 122:196.

22. Lindahl, K. F. 1979. Unrestricted killer cells recognize an antigen controlled by a gene linked to Tla. *Immunogenetics.* 8:71.

23. Forman, J. 1979. H-2 Unrestricted cytotoxic T cell activity against antigens controlled by genes in the Qa-Tla region. *J. Immunol.* 123:2451.

24. Stanton, T. H., and L. Hood. 1980. Biochemical identification of the Qa-1 alloantigen. *Immunogenetics.* 11:309.

25. Hauptfeld, V., M. Hauptfeld, and J. Klein. 1974. Tissue distribution of I region-associated antigens in the mouse. *J. Immunol.* 113:181.

26. Lake, P., and T. C. Douglas. 1978. Recognition and genetic control of helper determinants for cell surface antigen Thy-1. *Nature (Lond.).* 275:220.

27. Wernet, D., and F. Lilly. 1975. Genetic regulation of the antibody response to H-2D^b alloantigens in mice. I. Difference in activation of helper T cells in C57BL/10 and BALB/c congenic strains. *J. Exp. Med.* 141:573.

28. Mitchison, N. A. 1971. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. *Eur. J. Immunol.* 1:18.

29. Hamaoka, T., H. Fujiwara, K. Teshima, H. Aoki, H. Yamamoto, and M. Kitagawa. 1979. Regulatory functions of hapten-reactive helper and suppressor T lymphocytes. III. Amplification of a generation of tumor-specific killer T-lymphocyte activities by suppressor T-cell-depleted hapten-reactive T lymphocytes. *J. Exp. Med.* 149:185.

30. Green, W. R., R. C. Nowinski, and C. S. Henney. 1979. The generation and specificity of cytotoxic T cells raised against syngeneic tumor cells bearing AKR/Gross murine leukemia virus antigens. *J. Exp. Med.* 150:51.

31. Finberg, R., S. J. Burakoff, H. Cantor, and B. Benacerraf. 1978. Biological significance of alloreactivity: T cells stimulated by sendai virus-coated syngeneic cells specifically lyse allogeneic target cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:5145.

32. Fujiwara, H., and G. M. Shearer. 1980. Studies on *in vivo* priming of TNP-reactive cytotoxic effector system. II. Augmented secondary cytotoxic response by radioresistant T cells and demonstration of differential helper activity as a function of the stimulating dose of TNP-self. *J. Immunol.* 124:1271.

33. Ashman, R. B., and A. Mullbacher. 1979. A T helper cell for anti-viral cytotoxic T-cell responses. *J. Exp. Med.* 150:1277.

34. Martinez-A, C., and A. Coutinho. 1981. B-cell activation by helper cells is a two-step process. *Nature (Lond.).* 290:60.

35. Swain, S. L., G. Dennert, J. F. Warner, and R. W. Dutton. 1981. Culture supernatants of a stimulated T-cell line have helper activity that acts synergistically with interleukin 2 in the response of B cells to antigen. *Proc. Natl. Acad. Sci. U. S. A.* 78:2517.

36. Keller, D. M., J. E. Swierkosz, P. Marrack, and J. W. Kappler. 1980. Two types of functionally distinct, synergizing helper T cells. *J. Immunol.* 124:1350.