

BOTH L3T4⁺ AND Lyt-2⁺ HELPER T CELLS INITIATE
CYTOTOXIC T LYMPHOCYTE RESPONSES AGAINST
ALLOGENEIC MAJOR HISTOCOMPATIBILITY ANTIGENS
BUT NOT AGAINST TRINITROPHENYL-MODIFIED SELF

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The initiation of immune responses to nearly all soluble antigens requires the activation by accessory cells of class II-restricted helper T (Th)¹ cells (1, 2). In contrast, the role of Th cells in initiating immune responses to membrane antigens, especially to major histocompatibility complex (MHC)-encoded antigens, remains ill-defined and largely a matter of speculation. Since immune responses against MHC antigens are among the most potent of all immune responses, it is of special interest to identify the cellular interactions by which they are initiated (3). We and others have reported (4–8) that cytotoxic T lymphocyte (CTL) responses against class I alloantigens, like responses to soluble antigens, require a T cell-accessory cell interaction for their initiation. Surprisingly, however, we found (7, 8) that the T cells triggered by accessory cells in class I allospecific CTL responses were of two distinct restriction specificities, in that they could be either class II-restricted or unrestricted.

This study was undertaken to document the specificity, function, and Lyt phenotype of the T cells whose activation by accessory cells is a necessary early event in the generation of CTL responses against class I MHC antigens. That such T cells were Th cells was documented by synergy experiments in which they provided help for the activation of allospecific precursor CTL (pCTL). We found that there are two distinct subpopulations of Th cells that initiate responses to allogeneic class I MHC antigens: (a) an L3T4⁺,Lyt-2⁻ subset specific for class II accessory cell MHC determinants, and (b) an L3T4⁻,Lyt-2⁺ subset specific for class I accessory cell MHC determinants. Both L3T4⁺ and Lyt2⁺ Th cell subsets mediate their helper function, at least in part, by secreting soluble interleukin 2 (IL-2). Finally, L3T4⁻,Lyt-2⁺ Th cells initiate responses against allogeneic class

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¹ *Abbreviations used in this paper:* Con A SN, concanavalin A-stimulated spleen cell supernatant; CTL, cytotoxic T lymphocytes; E:T, effector/target; IL-2, interleukin 2; mAb, monoclonal antibody; MHC, major histocompatibility complex; pCTL, cytotoxic T lymphocyte precursors; Th, helper T cell; TNP, trinitrophenyl.

I MHC determinants, but do not initiate responses against trinitrophenyl (TNP)-modified self class I MHC determinants, even though many Lyt-2⁺ CTL effectors crossreactively recognize both allogeneic and TNP-self class I MHC determinants (9). Our study identifies an Lyt-2⁺ Th cell population that appears to be involved primarily in MHC-specific immune responses.

Materials and Methods

Animals. The MHC (K, I, D) alleles of the mouse strains used in this study are shown in each table. Mice were purchased from The Jackson Laboratory, Bar Harbor, ME, or were bred in our own animal colony.

Radiation Bone Marrow Chimeras. Radiation bone marrow chimeras are designated as bone marrow donor → irradiated recipient. An extensive description of the production and typing of such chimeras has been given elsewhere (10, 11). Briefly, recipient mice were irradiated with 950 rad from a ¹³⁷Cs source, and were reconstituted 2–6 h later with 1.5×10^7 bone marrow cells that had been depleted of T cells by pretreatment with a rabbit anti-mouse brain antiserum, a reagent specifically cytotoxic for all T cells (12), plus guinea pig complement. Spleen cells were obtained from each chimera no earlier than 2 mo after irradiation and bone marrow reconstitution, at which time they were >98% of donor bone marrow origin, as assessed by indirect immunofluorescence, and were tolerant of donor and host MHC determinants, as assessed by cell-mediated lysis and mixed lymphocyte proliferation (10, 11).

Monoclonal Antibodies (mAb). Anti-I-A^b mAb was a mouse ascites of the hybridoma cell line 25-9-17 described by Ozato and Sachs (13), and was used at a final concentration in culture of 2% (vol/vol). Anti-Lyt-2.2 mAb was a culture supernatant of the hybridoma cell line 83-12-5 produced by Dr. J. Bluestone, NIH. Anti-L3T4a mAb (14) was a culture supernatant of the hybridoma cell line GK1.5 generously supplied by Dr. F. Fitch, University of Chicago, IL. Anti-murine IL-2 receptor mAb were mouse ascites of the hybridoma cell lines 7D4 and 3C7 (15, 16).

Monoclonal Accessory Cells. M12.4.1 is an H-2^d, Ia⁺ B lymphoblastoid cell line with known accessory function (17). M12.C3 is an Ia⁻ variant cell line derived from M12.4.1 by γ -irradiation and negative immunoselection. The M12.C3 cell line continues to express H-2K^d, D^d, and L^d, but does not express detectable amounts of either I-A- or I-E-encoded determinants on the cell surface.²

Exogenous Helper Factor. As a source of soluble Th factors, 18-h culture supernatant from concanavalin A-stimulated BALB/c spleen cells (Con A SN) was used (18). The Con A SN was supplemented with 0.2 M α -methyl-D-mannoside to neutralize the excess Con A, and was used in culture at a final concentration of 12.5% (vol/vol).

Preparation of Cells. Responder, stimulator, and accessory cell populations were obtained from the spleens of unprimed mice. Where indicated, responder and stimulator populations were depleted of accessory cells by passage over Sephadex G-10 columns as previously described (19). Stimulator cells were irradiated with 2,000 rad. Unless otherwise indicated, accessory cells were spleen cells that had been depleted of T cells by pretreatment with rabbit anti-mouse brain antiserum plus guinea pig complement, and then irradiated with 2,000 rad. TNP modification of stimulator cells was performed where indicated with 10 mM trinitrobenzene sulfonate, as previously described (20).

Antibody-plus-complement Treatment of Responder Cells. Depletion of L3T4⁺ cells or Lyt-2⁺ cells was accomplished by incubating spleen cells at a density of 10^7 cells/ml with anti-L3T4 mAb (1:2 dilution of culture supernatant) or anti-Lyt-2.2 mAb (1:50 dilution of culture supernatant), respectively, for 30 min, on ice. Cells were then pelleted, resuspended, and incubated with rabbit complement for 30 min at 37°C. Treated cells were washed three times before culture. Efficacy of each treatment (>99%) was confirmed by immunofluorescence and flow microfluorometry (data not shown).

² Glimcher, L. H., D. J. McKean, E. Choi, and J. G. Seidman. Complex regulation of class II gene expression: Analysis with class II mutant cell lines. Manuscript submitted for publication.

In Vitro Generation of CTL. CTL were generated in either 0.2-ml microcultures or 2-ml macrocultures. 0.2-ml microcultures (96-well Nunclon; Nunc, Roskilde, Denmark) consisted of 4×10^5 responder cells, 4×10^5 stimulator cells, and, where indicated, 4×10^5 accessory cells. 2-ml macrocultures (Costar, Cambridge, MA.) consisted of 4×10^6 of each cell population. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and L-glutamine in a 7.5% CO₂-humidified air at 37°C for 5 d. Target cells were ⁵¹Cr-labeled Con A-induced splenic blast cells. For experiments performed in microculture, 4×10^3 target cells were directly added to each microculture on day 5. After 4-h incubation, percent specific lysis per microculture was determined. For experiments performed in macroculture, effector cells were harvested on day 5, and graded numbers were incubated with 4×10^3 target cells for 4 h. Results are expressed as percent specific lysis at an indicated effector/target (E:T) ratio. Percent specific lysis of target cells was calculated as follows: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Each point represents the mean percent specific ⁵¹Cr release from three or four replicate wells. Standard errors were always <5% of the mean value, and are omitted.

Assay for IL-2 Activity. Responder cells (4×10^5) and stimulator cells (4×10^5) were cultured together in 0.2-ml round-bottom microcultures in the presence of 0.01% ascites of anti-murine IL-2 receptor mAb (7D4) to inhibit IL-2 consumption during the culture period. After 4 d, 0.1 ml of supernatant was removed from each well and assayed for its ability to maintain the growth of the IL-2-dependent cell line, HT-2. The amount of 7D4 mAb present in the culture supernatants was insufficient to interfere with the IL-2-dependent growth of HT-2 cells (15, 16) because of the large number of IL-2 receptors these cells express (our unpublished observation). After 24 h of incubation at 37°C in 5% CO₂, cultures of 4×10^3 HT-2 cells plus culture supernatant were pulsed with 1 μCi [³H]thymidine, and harvested 12–18 h later. Results are expressed as the amount of [³H]thymidine incorporated by HT-2 cells in response to the culture supernatants. Each point represents the arithmetic mean cpm of quadruplicate cultures.

Results

Role of Class I- and Class II-restricted T Cell-Accessory Cell Interactions in the Induction of Allospecific CTL Responses. It was previously determined that there are two general categories of T cell-accessory cell interactions that initiate primary class I allospecific CTL responses (7). The first category involves responder T cell recognition of class II MHC determinants, and the second category involves responder T cell recognition of class I MHC determinants that are expressed by accessory cells. Table I provides an example of these two distinct categories of cell interactions. Anti-D^d CTL responses were generated in vitro by stimulating B10 spleen cells (designated by their K,I,D alleles as b,b,b) with B10.YBR (b,b,d) stimulator spleen cells (Table I, group 1). This response was strictly dependent on the presence of accessory cells, and so was abrogated by passing both responder and stimulator cell populations over Sephadex G-10 columns (Table I, group 2). This CTL response could be reconstituted by the addition to culture of accessory cells of either responder (b,b,b) or stimulator (b,b,d) type (Table I, groups 3 and 5). However, the responses obtained in these two cases involved different categories of T cell-accessory cell interaction, as indicated by differences in the effect of anti-Ia mAb (Table I, groups 4 and 6). Consistent with results reported in detail previously (7, 8), responses mediated by responder-type accessory cells were completely blocked by anti-Ia mAb, whereas responses mediated by stimulator-type accessory cells were largely unaffected by anti-Ia mAb, even though the anti-Ia mAb could bind equally well

TABLE I
Distinct T Cell–Accessory Cell Interactions Can Initiate Allospecific CTL Responses

Group	Treatment of responder B10 (b,b,b) cells	Treatment of stimulator B10.YBR (b,b,d) cells*	Haplotype of added accessory cells	Antibody in culture [‡]	Percent specific lysis of target (b,b,d) cells in micro-culture
1	untreated	untreated	—	—	79
2	G-10	G-10	—	—	0
3	G-10	G-10	b,b,b (B10)	—	35
4	G-10	G-10	b,b,b (B10)	anti-I-A ^b	0
5	G-10	G-10	b,b,d (B10.YBR)	—	73
6	G-10	G-10	b,b,d (B10.YBR)	anti-I-A ^b	64

* Stimulator cell antigen was D^d.

[‡] 2% (vol/vol) final concentration in culture of 25-9-17 ascites.

to both types of accessory cells (Table I, groups 4 and 6). Thus, responses mediated by responder-type accessory cells (which do not express any foreign class I MHC determinants) were initiated entirely by class II–restricted T cells, while responses mediated by stimulator-type accessory cells (which do express foreign class I MHC determinants) were initiated by T cells that appeared to be class II–unrestricted and putatively specific for the allogeneic class I MHC determinants expressed by the accessory cells.

To further assess the possibility that accessory cell activation of putatively class I–specific, Ia–unrestricted T cells did not involve recognition of accessory cell Ia determinants, an experiment was performed using a monoclonal cell line that was Ia[–], but which potentially possessed accessory function. M12.C3 is an irradiation-induced Ia[–] variant of the M12.4.1 lymphoblastoid cell line previously shown to have accessory function (17). In contrast to the parental M12.4.1 line, which is H-2^d and Ia⁺, M12.C3 is H-2^d but Ia[–], in that it expresses neither I-A nor I-E–encoded determinants on its cell surface. On the presumption that the mutation resulting in the loss of Ia expression did not also necessarily result in the loss of accessory function, the Ia[–] M12.C3 and the parental M12.4.1 lines were used as accessory cells in allospecific CTL responses (Table II). It can be seen that the Ia[–] M12.C3 cell line did provide accessory function in allospecific CTL responses, but only when it was of stimulator type (Table II, group 6), not when it was of responder type (Table II, group 12). As would be expected, neither the ability of the Ia[–] M12.C3 cell line nor the ability of the parental M12.4.1 cell line to provide accessory function when they were of stimulator type was blocked by the addition to culture of anti-I^d mAb (data not shown). The failure of Ia[–] M12.C3 cells to mediate responses when they were of responder type and so did not express allogeneic class I MHC determinants is (a), consistent with the fact that responses mediated by responder-type accessory cells necessarily involve an Ia–restricted T cell–accessory cell interaction, (b) demonstrates that M12.C3 cells do not simply secrete nonspecific T cell–activating factors, and (c) argues against the possibility that M12.C3 cells are induced during culture to express functionally relevant but undetectable quantities of Ia determinants on their cell surface. More importantly, the success of Ia[–] M12.C3 cells in mediating

TABLE II
Activation of Ia-Unrestricted T Cells by Mutant Ia⁻ Cell Line with Accessory Function

Group	Treatment of responder B10.OL (d,d,k) cells	Treatment of stimulator B10.D2 (d,d,d) cells*	Haplotype of added accessory cells	Percent specific lysis of target cells at E:T ratio of 50:1	
				d,d,d	d,d,k
1	untreated	untreated	—	59	-7
2	G-10	G-10	—	-8	-7
3	G-10	G-10	d,d,d (B10.D2)	64	-6
4	G-10	G-10	d,d,k (B10.OL)	53	-8
5	G-10	G-10	d,d,d (M12.4.1) [‡]	46	-3
6	G-10	G-10	d,-,d (M12.C3) [‡]	44	-4
	B10.D2 (d,d,d)	B10.OL (d,d,k) [§]			
7	untreated	untreated	—	-7	65
8	G-10	G-10	—	-8	-2
9	G-10	G-10	d,d,d (B10.D2)	-6	59
10	G-10	G-10	d,d,k (B10.OL)	-4	65
11	G-10	G-10	d,d,d (M12.4.1)	-2	60
12	G-10	G-10	d,-,d (M12.C3)	-7	5

* Stimulator cell antigen was D^d.

[‡] 2×10^5 cells/culture.

[§] Stimulator cell antigen was D^k.

responses when they were of stimulator type, and so expressed allogeneic class I MHC determinants, strongly supports the contention that the T cells that are directly activated by stimulator-type accessory cells are not only Ia-unrestricted, but are Ia-independent. These Ia-independent T cells must be specific for allogeneic class I MHC determinants, since there exists only a class I difference between B10.OL (d,d,k) responder T cells, which were triggered by M12.C3 cells, and B10.D2 (d,d,d) responder T cells, which were not triggered by them. Moreover, this experiment demonstrates that even though accessory cells are generally Ia⁺, including those normally involved in the activation of Ia-independent class I-restricted T cells (7), the expression of accessory function does not, in itself, require the cell surface expression of Ia determinants.

L3T4⁺ Th Cells in Allospecific CTL Responses. Although exceptions exist, L3T4 appears to be expressed primarily on class II-restricted T cells, whereas Lyt-2 appears to be expressed primarily on class I-restricted T cells (21). Consequently, we wished to assess the role of L3T4⁺ T cells in initiating class I allospecific CTL responses by each type of T cell-accessory cell interaction. It was of particular interest to determine the cell surface phenotype of T cells triggered by responder-type accessory cells, since it was shown previously (8) that these T cells are specific for shed class I alloantigens presented in association with self-class II accessory cell determinants. Table III shows that the addition to culture of anti-L3T4 mAb significantly inhibited anti-D^d CTL responses mediated by either responder-type or class II-disparate accessory cells (Table III, groups 3-6), but had no effect on responses mediated by accessory cells expressing the stimulatory class I alloantigen (Table III, groups 7 and 8). Similar results were obtained over

TABLE III
Ia-restricted T-Accessory Cell Interactions Are Mediated by L3T4⁺ Cells

Group	Treatment of responder B10 (b,b,b) cells	Treatment of stimulator B10.QBR (b,b,q) cells*	Haplotype of added accessory cells	Antibody in culture (25% vol/vol)	Percent specific lysis of target cells at E:T ratio of 40:1	
					b,b,q	b,b,b
1	untreated	untreated	—	—	67	3
2	G-10	G-10	—	—	2	-1
3	G-10	G-10	b,b,b (B10)	—	27	1
4	G-10	G-10	b,b,b (B10)	L3T4a	-1	0
5	G-10	G-10	b,bm12,b (bm12)	—	63	4
6	G-10	G-10	b,bm12,b (bm12)	L3T4a	16	-1
7	G-10	G-10	b,b,q (B10.QBR)	—	53	0
8	G-10	G-10	b,b,q (B10.QBR)	L3T4a	55	2

* Stimulator cell antigen was D^a.

a 10-fold (2.5–25% final concentration) titration of anti-L3T4 mAb. Thus, these results demonstrate that L3T4⁺ T cells are involved in the initiation of class I allospecific CTL responses, at least in those responses mediated by accessory cells that do not express foreign class I alloantigens. This result is concordant with previous observations (7) that such responses require the activation of class II-restricted T cells (Tables I and II).

To conclude that the L3T4⁺ T cells involved in allospecific CTL responses are in fact Th cells, it was necessary to show that these cells would provide help for the activation of allospecific pCTL. In Fig. 1, B10 responder cells were cultured with B10.QBR stimulators to generate anti-D^a CTL. The response (48% lysis of B10.QBR target cells) was abrogated by G-10-passage of both responder and stimulator cells (3% lysis), and was reconstituted by the addition to culture of either class II-disparate bm12 (b,bm12,b) accessory cells (Fig. 1, *left*) or class I-disparate B10.QBR (b,b,q) accessory cells (Fig. 1, *right*). L3T4⁺ cells were required only in the response mediated by bm12 accessory cells, but not B10.QBR accessory cells (Fig. 1, group 2). Since allogeneic I-A^{bm12} accessory cell determinants are potent stimulators of L3T4⁺ H-2^b Th cells, this result shows that the responder T cell population was the only source of functional L3T4⁺ Th cells in the response cultures, and that responses mediated by class I-disparate D^a accessory cells did not require L3T4⁺ Th cells. In contrast, Lyt-2⁺ cells were required regardless of the accessory cell involved (Fig. 1, group 3), because primary class I allospecific pCTL are uniformly L3T4⁻,Lyt-2⁺. Indeed, the Lyt phenotype of class I allospecific CTL effectors was found to be L3T4⁻,Lyt-2⁺, regardless of the type of T cell-accessory cell interaction involved in their generation (data not shown). Thus, for responses initiated by a class II-restricted T cell-accessory cell interaction (Fig. 1, *left*), L3T4⁻,Lyt-2⁺ T cells constitute a population of pCTL (Fig. 1, group 2) and L3T4⁺,Lyt-2⁻ T cells constitute a population of putative Th cells (Fig. 1, group 3). That L3T4⁺,Lyt-2⁻ T cells function as Th cells in class I allospecific CTL responses is shown in Fig. 1, group 4, in which they showed synergy with, and provided help for the activation of L3T4⁻,Lyt-2⁺ pCTL.

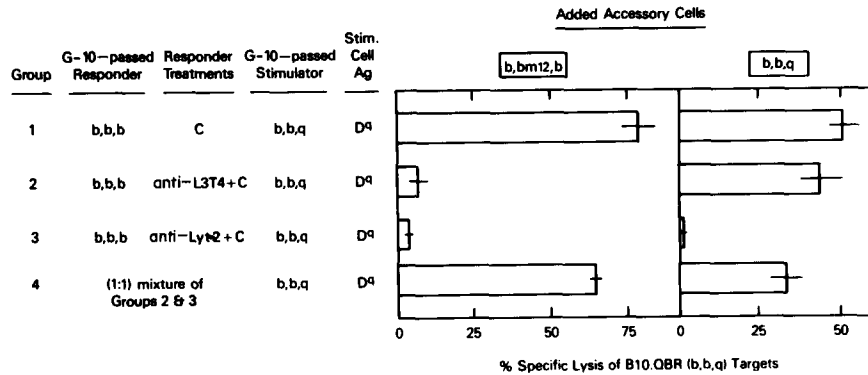


FIGURE 1. L3T4⁺ Th cells in allospecific CTL responses. B10 (b,b,b) responder cells and B10.QBR (b,b,q) stimulator cells were depleted of accessory cells by G-10 passage, and cultured with either class II-disparate bm12 (b,bm12,b) accessory cells (*left*) or class I-disparate B10.QBR accessory cells (*right*). The B10 responding cells were further treated with complement (C) anti-L3T4 plus C, or anti-Lyt-2 plus C, as indicated. This experiment was performed in microculture, and the results are expressed as percent specific lysis per microculture.

It can be concluded from Table III and Fig. 1 that, in class I allospecific CTL responses, the T cells activated by an Ia-restricted interaction with accessory cells are (a) L3T4⁺,Lyt-2⁻, and (b) function as Th cells for the activation of class I allospecific pCTL, which express the reciprocal Lyt phenotype. It can be further concluded that, in contrast to CTL responses mediated by accessory cells expressing self class I MHC determinants, CTL responses mediated by accessory cells expressing allogeneic class I MHC determinants do not require L3T4⁺ T cells, and can be mediated entirely by L3T4⁻,Lyt-2⁺ T cells (Fig. 1, *right*).

Lyt-2⁺ Th Cells in Allospecific CTL Responses. Since CTL responses mediated by accessory cells expressing allogeneic class I MHC determinants can be mediated entirely by L3T4⁻,Lyt-2⁺ T cells (Fig. 1, *right*), putative Th and pCTL populations participating in this response cannot be separated into distinct T cell subpopulations by differences in their Lyt phenotype. Nevertheless, since synergy is the classical method of defining a helper-effector cell interaction, it was necessary to design experiments capable of detecting synergy between putative Lyt-2⁺, class I-restricted Th, and Lyt-2⁺, class I-restricted pCTL subpopulations.

The potential participation of Lyt-2⁺ Th cells in allospecific CTL responses was first assessed by determining whether Lyt-2⁺ T cells with one recognition specificity could provide help for the activation of Lyt-2⁺ pCTL with a different recognition specificity. In Table IV, Lyt-2⁺ T cells specific for allogeneic D^d accessory cell determinants were assessed for their potential to show synergy with, and provide help for the activation of Lyt-2⁺ minor-H-specific pCTL (22). In vivo-immunized B10 responder spleen cells were depleted of both accessory cells and L3T4⁺ T cells, then cultured with G-10-passed C3H.SW stimulator cells. In the absence of any added accessory cells, no anti-minor H-specific CTL were generated, as indicated by their failure to lyse C3H.SW target cells (Table IV, group 1). In addition, no anti-minor H CTL were generated upon the addition of responder-type B10 accessory cells (group 3), because the responder population was devoid of L3T4⁺, Ia-restricted Th cells. In contrast, anti-minor

H CTL were successfully generated upon addition of B10.YBR (b,b,d) accessory cells, which express allogeneic D^d determinants (group 4), indicating that D^d-specific Lyt-2⁺ T cells had been synergistic with, and provided help for C3H-minor H-specific pCTL. The anti-minor H CTL effectors generated in group 4 must have been derived from an Lyt-2⁺ subpopulation that was distinct from the Th cells with which they showed synergy, because the Lyt-2⁺ B10 responding cell population did not contain detectable numbers of T cells that crossreactively recognized both D^d and C3H-minor H alloantigens (Table IV, groups 2 and 6). Thus, this experiment indicates that the T cells activated by recognition of allogeneic class I accessory cell MHC determinants are L3T4⁻,Lyt-2⁺ and able to function as Th cells for the activation of Lyt-2⁺ pCTL.

Because the participation of Lyt-2⁺ class I-restricted Th cells in immune responses has not been generally appreciated, a second type of experiment was performed. In this experiment, responder T cells from fully allogeneic radiation bone marrow chimeras were used because they provided a potential tool for physically separating the interacting class I-restricted Th and pCTL populations (Fig. 2). It has previously been documented (10, 11, 23) that T cells from fully allogeneic chimeras (*a*) are of donor origin, (*b*) are tolerant to both donor and host MHC determinants, and (*c*) behave as though they are functionally blind to donor Ia determinants, in that they fail to recognize donor Ia determinants as either foreign or self. Thus, H-2^b T cells from b → k and b → d chimeras are similar in that both are blind to I^b determinants. However, H-2^b T cells from these chimeras differ in that T cells from b → k chimeras are tolerant to H-2^k but not H-2^d determinants (Fig. 2, groups 5 and 6), whereas T cells from b → d chimeras are tolerant to H-2^d but not H-2^k determinants (Fig. 2, groups 7 and 8). Consequently, D^k-specific pCTL would only be present in T cell populations from b → d chimeras, whereas D^d-specific Th cells would only be present in T cell populations from b → k chimeras. Indeed, neither chimeric T cell population by itself was able to generate an anti-D^k CTL response in the presence of B10.YBR (b,b,d) accessory cells (Fig. 2, *left*, groups 1 and 2). However, when both chimeric T cell populations were cultured together, T cells from b → k chimeras specific for D^d accessory cell determinants showed synergy with, and provided help for the activation of anti-D^k pCTL from b → d chimeras (Fig. 2,

TABLE IV
L3T4⁻,Lyt-2⁺ T Cells Can Function as Class I-Restricted Th Cells

Responder group*	Stimulator [‡]	Added accessory cells	Stimulator cell antigen	Accessory cell antigen	Percent specific lysis of targets at various E:T ratios				
					C3H.SW		B10.YBR		B10
					40	20	40	20	40
1	C3H.SW	—	Minor H	—	-1	-1	-2	-2	-2
2	C3H.SW	Con A SN	Minor H	—	61	50	-1	-2	2
3	C3H.SW	B10	Minor H	—	8	5	-2	-1	-1
4	C3H.SW	B10.YBR	Minor H	D ^d	33	23	38	28	-2
5	—	B10.YBR	—	D ^d	2	2	32	24	-4
6	B10.YBR	Con A SN	D ^d	—	2	0	54	42	-2

* G-10-passed responder cells were treated with anti-L3T4a + C before use, and were taken from B10 mice primed intraperitoneally with 2×10^7 C3H.SW spleen cells 2 wk previously.

[‡] G-10-passed stimulator cells.

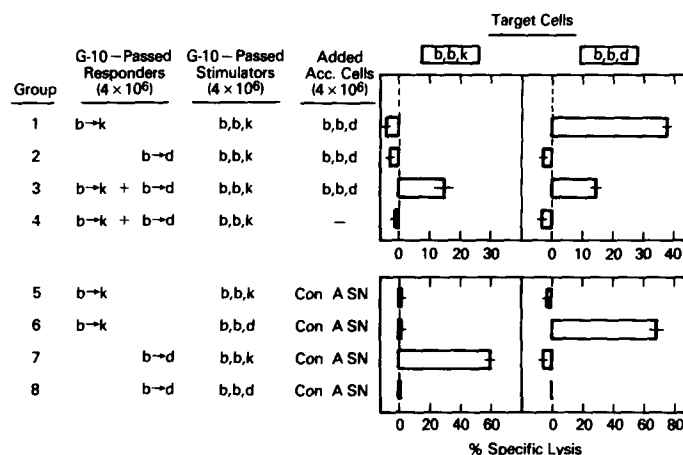


FIGURE 2. Lyt-2^+ Th cells in allospecific CTL responses. $\text{B10} \rightarrow \text{B10.BR}$ ($b \rightarrow k$) or $\text{B10} \rightarrow \text{B10.D2}$ ($b \rightarrow d$) allogeneic chimeric responder cells and C3H.KBR (b,b,k) stimulator cells were depleted of accessory cells by G-10 passage, and cultured with B10.YBR (b,b,d) accessory cells. Each macroculture contained a total of 4×10^6 responder cells, so cultures containing two responder cell populations (groups 3 and 4) contained only 2×10^6 of each population. Each culture was assayed for both anti- D^k and anti- D^d CTL. Results represent percent specific lysis at a 40:1 E:T ratio.

left, group 3). The response observed in this group when the two chimeric responder cell populations were cultured together reflects synergy between two separate but interacting class I-restricted T cell populations, because a single population of class I-restricted T cells that would crossreactively recognize both D^d and D^k determinants was deleted from each chimeric T cell population. Indeed, such crossreactive T cells are not detected in either chimera population, even in the presence of Con A SN (Fig. 2, groups 5–8).

Thus, these experiments demonstrate that, in class I allospecific CTL responses, the Ia-independent T cells that are triggered by the recognition of allogeneic class I accessory cell MHC determinants are L3T4^+ , Lyt-2^+ , and so cannot be distinguished by Lyt phenotype from pCTL, but can be shown to function as Lyt-2^+ Th cells for the activation of other Lyt-2^+ allospecific pCTL. It is not yet known whether these Lyt-2^+ Th cells are dual-function cells able also to differentiate into CTL effectors themselves (24, 25).

Role of IL-2 Secretion by Th Cells in the Generation of Allospecific CTL Responses. It seemed likely that at least one of the mechanisms by which L3T4^+ and Lyt-2^+ Th cells provided help for the activation of allospecific pCTL involved the secretion by the Th cells of soluble lymphokines such as IL-2. Consequently, we next determined whether the activation of allospecific pCTL required the binding of soluble IL-2 to their cell surface IL-2 receptors. In Table V, mAb specific for the murine IL-2 receptor were assessed for their ability to block the generation of allospecific CTL responses. It should be noted that anti-IL-2 receptor mAb such as 7D4 and 3C7 do not interfere with the secretion of IL-2 by either L3T4^+ or Lyt-2^+ Th cells (Mizuochi, T., T. Malek, and A. Singer, manuscript in preparation). It can be seen in Table V that anti-IL-2 receptor mAb either abrogated or significantly inhibited allospecific CTL responses

TABLE V
Anti-IL-2 Receptor Antibodies Block the In Vitro Generation of Allospecific CTL Responses

Group	Treatment of responder B10 (b,b,b) cells	Treatment of stimulator B10.QBR (b,b,q) cells*	Haplotype of added accessory cells	Antibody in culture [‡]	Percent specific lysis of target cells at E:T ratio of 20:1	
					b,b,q	b,b,b
1	untreated	untreated	—	—	62	3
2	G-10	G-10	—	—	1	1
3	G-10	G-10	b,b,b (B10)	—	21	1
4	G-10	G-10	b,b,b (B10)	7D4 + 3C7	-2	-3
5	G-10	G-10	b,bm12,b (bm12)	—	69	4
6	G-10	G-10	b,bm12,b (bm12)	7D4 + 3C7	22	0
7	G-10	G-10	b,b,q (B10.QBR)	—	44	0
8	G-10	G-10	b,b,q (B10.QBR)	7D4 + 3C7	5	0

* Stimulator cell antigen was D^a.

[‡] 0.01% (vol/vol) of 7D4 ascites and 0.5% (vol/vol) of 3C7 ascites in culture.

initiated by either class II-restricted T cell-accessory cell interactions (Table V, groups 3–6) or class I-restricted T cell-accessory cell interactions (Table V, groups 7 and 8). Thus, regardless of the T cell-accessory cell interaction involved, the presence in the response culture of soluble IL-2 is necessary for the activation of class I allospecific pCTL. It is reasonable to deduce from this result that, for allospecific CTL responses, each T cell-accessory cell interaction results in the triggering by accessory cells of T cells to secrete soluble IL-2.

To test this deduction directly, response cultures were simultaneously assayed for both CTL generation and IL-2 production. To detect IL-2 production in these cultures, we found that it was necessary to inhibit the consumption of IL-2 during the culture period, and that this could be accomplished by the addition of anti-IL-2 receptor mAb (7D4) (Mizuochi, T., T. Malek, and A. Singer). However, since anti-IL-2 receptor mAb block the activation of pCTL (Table V), 7D4 mAb was not added to cultures that were to be assayed for CTL generation, but instead was added only to parallel cultures that were assayed only for IL-2 production.

In Table VI, B10 → B10.BR (b,b,b → k,k,k) chimeric responder cells were cultured with D^a-disparate B10.QBR (b,b,q) stimulator cells, and parallel cultures were then assayed either for CTL or IL-2 activity. In terms of CTL activity, the anti-D^a CTL response could be mediated by either class II-disparate bm12 (b,bm12,b) accessory cells, or by class I-disparate B10.QBR (b,b,q) accessory cells, but could not be mediated by donor type B10 (b,b,b) accessory cells (Table VI, groups 3–5). From both the present and previous studies (7), donor-type accessory cells would not be expected to mediate this CTL response, because they would be unable to activate any Th cells in the chimeric responder population. That is, donor type accessory cells express only syngeneic class I MHC determinants, and so would be unable to trigger any class I-restricted, Lyt-2⁺ allospecific responder Th cells, and such donor cells express only donor-type I^b determinants, to which the chimeric responder T cells are blind and so would

TABLE VI
Correlation Between IL-2 Production and CTL Generation in Allospecific CTL Responses

Group	Treatment of responder B10 → B10.BR (b,b,b → k,k,k) cells	Treatment of stimulator B10.QBR (b,b,q) cells*	Haplotype of added accessory cells	CTL activity [‡]	IL-2 activity [§]
1	untreated	untreated	—	69	12.4
2	G-10	G-10	—	3	0.6
3	G-10	G-10	b,b,b (B10)	2	0.2
4	G-10	G-10	b,bm12,b (bm12)	68	32.7
5	G-10	G-10	b,b,q (B10.QBR)	63	20.2

* Stimulator cell antigen was D^q.

[‡] Percent specific lysis per microculture, using B10.QBR (b,b,q) targets.

[§] As measured by [³H]thymidine incorporation (cpm × 10⁻³) by HT-2 cell line.

be unable to trigger any class II-restricted L3T4⁺ responder Th cells. Indeed, no IL-2 was detected in the supernatants of parallel cultures containing donor-type accessory cells (Table VI, group 3). In contrast, class II-disparate bm12 accessory cells would be expected to successfully mediate this allospecific CTL response because they would be able to trigger L3T4⁺, I-A^{bm12}-specific Th cells, and IL-2 was detected in the supernatants of parallel cultures containing bm12 accessory cells (group 4). Class I-disparate B10.QBR (b,b,q) accessory cells would also be expected to mediate this allospecific CTL response, even though they express I^b determinants to which the chimeric responder Th cells are blind, because they would be able to trigger D^q allospecific Lyt-2⁺ Th cells. Indeed, IL-2 was detected in the supernatants of parallel cultures containing B10.QBR (b,b,q) accessory cells (Table VI, group 5). Thus, allospecific CTL were generated only in those response cultures in which Th cells were triggered by accessory cells to secrete IL-2.

Taken together, these data indicate that one of the roles performed by Th cells in allospecific CTL responses is to secrete IL-2, and that the binding by pCTL of IL-2 to their IL-2 receptors is necessary for the generation and/or expansion of primary allospecific CTL effectors.

Only L3T4⁺ Th Cells Participate in CTL Responses Against TNP-Self Determinants. A high proportion of CTL specific for allogeneic class I MHC determinants crossreactively recognize TNP-modified self class I MHC determinants (9). Since many Lyt-2⁺ CTL recognize both TNP-modified self and allogeneic class I MHC determinants, it seemed reasonable to expect that the Th cells (especially the Lyt-2⁺ Th cells), which initiate CTL responses against allogeneic class I MHC determinants, would also initiate CTL responses against TNP-modified self class I MHC determinants. To examine this possibility, B10 (b,b,b) T cells were cultured with TNP-self or B10.YBR (b,b,d) stimulator cells to generate CTL specific for either TNP-H-2^b or D^d determinants (Table VII). To examine the role of L3T4⁺ and Lyt-2⁺ Th cells in anti-TNP CTL responses, anti-L3T4 mAb was added to the response cultures. In marked contrast to CTL responses against allogeneic class I MHC determinants, CTL responses against TNP-self determinants were completely blocked by anti-L3T4 mAb (Table VII, compare groups 1 and 2 with 8 and 9), indicating that TNP-self CTL responses strictly require

TABLE VII
In Contrast to CTL Responses Against Allogeneic Class I Determinants, Primary CTL Responses Against TNP-Self Determinants Are Mediated Exclusively by L3T4⁺ Th Cells

Group	Treatment of responder B10 (b,b,b) cells	Treatment of stimulator TNP-B10 (b,b,b) cells*	Haplotype of added accessory cells	Antibody in culture [‡]	Percent specific lysis of target cells in microculture	
					TNP-b,b,b	b,b,b
1	untreated	untreated	—	—	55	0
2	untreated	untreated	—	anti-L3T4a	-2	-1
3	G-10	G-10	—	—	2	1
4	G-10	G-10	b,b,b (B10)	—	40	0
5	G-10	G-10	b,b,b (B10)	anti-L3T4a	-1	0
6	G-10	G-10	TNP-b,b,b (TNP-B10)	—	23	0
7	G-10	G-10	TNP-b,b,b (TNP-B10)	anti-L3T4a	-1	-2
B10.YBR (b,b,d) [§]						
8	untreated	untreated	—	—	65	-2
9	untreated	untreated	—	anti-L3T4a	45	0
10	G-10	G-10	—	—	1	-3
11	G-10	G-10	b,b,b (B10)	—	49	-2
12	G-10	G-10	b,b,b (B10)	anti-L3T4a	-1	-4
13	G-10	G-10	b,b,d (B10.YBR)	—	65	1
14	G-10	G-10	b,b,d (B10.YBR)	anti-L3T4a	55	0

* Stimulator cell antigen was TNP-self.

[‡] 25% (vol/vol) culture supernatant of GK1.5 hybridoma.

[§] Stimulator cell antigen was D^d.

L3T4⁺ Th cells. To further examine this point, the response cultures were depleted of accessory cells, and then reconstituted with either accessory cells of responder (b,b,b) type, which do not bear stimulatory TNP-self determinants (Table VII, group 4), or accessory cells of stimulator type (TNP-b,b,b), which do bear stimulatory TNP-self determinants (Table VII, group 6). Similar to allospecific CTL responses, TNP-self CTL responses mediated by accessory cells of responder type are initiated by L3T4⁺ Th cells, and so were inhibited by anti-L3T4 mAb (Table VII, compare groups 4 and 5 with 11 and 12). However, unlike allospecific CTL responses, TNP-self CTL responses mediated by accessory cells of stimulator type also require L3T4⁺ Th cells, and so were also inhibited by anti-L3T4 mAb (Table VII, compare groups 6 and 7 with 13 and 14). Thus, this experiment demonstrates that, in contrast to their ability to initiate allospecific CTL responses, Lyt-2⁺ Th cells are unable to initiate TNP-self CTL responses.

Discussion

This study characterizes the Th cells that initiate primary CTL responses against allogeneic and TNP-modified self class I MHC determinants. It shows that two distinct Th cell subsets participate in allospecific CTL responses: an L3T4⁺,Lyt-2⁻ Th cell subset specific for class II MHC determinants expressed

by accessory cells, and an L3T4⁻,Lyt-2⁺ Th cell subset specific for class I MHC determinants expressed by accessory cells. While both Th cell populations require accessory cells for their activation, the accessory cells, which trigger class I-restricted Lyt-2⁺ Th cell populations, do not need to be Ia⁺, as these Th cells were triggered by an Ia⁻ variant of a cell line with known accessory function. Both Th cell populations were documented as functioning as helper cells in allospecific CTL responses by their ability to collaborate with and activate allospecific pCTL. The mechanism by which these Th cells provide help to allospecific pCTL was shown to be due, at least in part, to their secretion of IL-2. Finally, despite the many similarities between primary allospecific and primary TNP-self CTL responses, we found that Lyt-2⁺ Th cells initiate allospecific CTL responses, but do not initiate TNP-self CTL responses.

We previously reported (7) that the initiation of class I allospecific CTL responses required an MHC-specific T cell-accessory cell interaction, and that these interactions were of three distinct specificities, involving: (a) responder T cell recognition of self class II accessory cell determinants, (b) responder T cell recognition of allogeneic class II accessory cell determinants, and (c) responder T cell recognition of allogeneic, but not self class I accessory cell determinants. Interestingly, the self class II-restricted T cells involved in the initiation of allospecific CTL responses were not autoreactive T cells specific for self class II accessory cell determinants per se, but rather were shown to be antigen-specific T cells that recognized a composite (class I plus class II) MHC determinant, consisting of shed and processed class I alloantigen, presented in association with self class II accessory cell determinants (8). Consequently, it was not clear what the Lyt phenotype of such T cells would be. We have shown that the Th cells specific for either self or allogeneic class II accessory cell MHC determinants were L3T4⁺,Lyt-2⁻. In other words, the Lyt phenotype of these class II-restricted Th cells was concordant with their restriction specificity (21), regardless of their nominal specificity for processed class I MHC determinants.

The existence of a class II-unrestricted T cell population that initiates primary allospecific CTL responses upon recognition of allogeneic class I accessory cell MHC determinants was reported previously (7). Herein, we show these class II-unrestricted T cells to be restricted to class I accessory cell MHC determinants, to be L3T4⁻,Lyt-2⁺, and to function as helper cells for the activation of allospecific pCTL. By using an Ia⁻ variant of a tumor cell line with known accessory function, it was also possible to show that the activation of these Lyt-2⁺ Th cells by accessory cells did not require the expression of Ia determinants on the accessory cell surface. Thus, as was the case for class II-restricted Th cells, the Lyt phenotype of class I-restricted Th cells was concordant with their MHC restriction specificity (21). While these Lyt-2⁺ cells were clearly able to function as Th cells for the activation of helper cell-dependent pCTL, we do not as yet know whether they function only as helper cells, or whether they are dual-function cells that also differentiate into CTL effector cells (24, 25).

It seemed likely that at least one of the functions performed by Th cells in allospecific CTL responses was the production and secretion of IL-2. That this is the case was shown by experiments demonstrating that: (a) Th cells in allospecific CTL responses do secrete IL-2 as a result of an MHC-specific

interaction with accessory cells, (*b*) the activation of allospecific pCTL requires that they bind IL-2 to a specific receptor on their cell surface, and (*c*) allospecific CTL are generated only in those response cultures in which IL-2 is also produced. However, given the possibility that Lyt-2⁺ Th cells are dual-function cells, it was somewhat surprising that anti-IL-2 receptor mAb not only inhibited allospecific CTL responses mediated by class II-restricted L3T4⁺ Th cells, but also inhibited allospecific CTL responses mediated by accessory cells that activated class I-restricted Lyt-2⁺ Th cells. Consequently, if Lyt-2⁺ Th cells are dual-function cells, they resemble conventional Th cells in that they secrete IL-2, and they resemble conventional pCTL in that they require IL-2 bound to their cell surface receptors in order to become cytolytic effector cells.

The ability of Lyt-2⁺ T cells to function as helper cells in immune responses was shown originally by Swain and colleagues (26, 27). However, they demonstrated the helper activity of Lyt-2⁺ T cells only for histoincompatible B cells, and only in circumstances in which the Lyt-2⁺ T cells had been pretreated with mitomycin C. Our study shows that a subset of Lyt-2⁺ T cells functions as helper cells for the activation of histocompatible pCTL in conventional class I allospecific CTL responses. In fact, Lyt-2⁺ T cells may be the predominant Th cells functioning in class I allospecific CTL responses, because such responses were largely unaffected by maneuvers that block the activation of L3T4⁺ Th cells, such as the addition to culture of anti-Ia or anti-L3T4 mAb (Tables I and III), and were even unaffected by the cytotoxic elimination of L3T4⁺ Th cells from the response cultures entirely (Fig. 1).

In contrast to immune responses against membrane-bound MHC alloantigens, Lyt-2⁺ Th cells have not been observed participating in immune responses against soluble antigens. Consequently, we wished to further identify the immune responses in which Lyt-2⁺ Th cells participate. It has long been appreciated that CTL responses against allogeneic class I MHC determinants and TNP-modified self class I determinants are very similar. For example, both can be generated in primary cultures, and the CTL effectors generated in each response extensively crossreact. Nevertheless, we found that, in contrast to primary allospecific CTL responses, primary TNP-self CTL responses are initiated exclusively by L3T4⁺,Lyt-2⁻ Th cells. This result is consistent with previous reports (28) that the initiation of primary CTL responses against TNP-modified self class I MHC determinants strictly requires responder T cell recognition of class II accessory cell MHC determinants. Thus, even though primary anti-TNP and anti-allo-CTL are Lyt-2⁺ and extensively crossreact, Lyt-2⁺ Th cells are unable to initiate primary TNP-self CTL responses.

The failure of class I-restricted Lyt-2⁺ Th cells to initiate anti-TNP CTL responses raises three provocative points. First, it raises the possibility that Lyt-2⁺ Th cells and Lyt-2⁺ pCTL express different receptor specificities, since they apparently differ in their recognition of TNP-modified self MHC determinants. Second, it suggests that Lyt-2⁺ Th cells may express a receptor repertoire that is specific primarily for allogeneic rather than antigen-modified self determinants. And finally, it raises the question of whether allospecific CTL effectors triggered by Lyt-2⁺, as opposed to L3T4⁺ Th cells, crossreactively lyse TNP-modified self targets. However, we have not yet detected any differences in the ability of

allospecific CTL generated by either L3T4⁺ or Lyt-2⁺ Th cells to crossreactively lyse TNP-self targets (our unpublished data), a result that is consistent with helper cell recruitment of pCTL by soluble mediators. All three of these points are currently under investigation.

It is now possible to reconsider an observation made by Burakoff et al. (29), which was interpreted as demonstrating fundamental differences in the differentiation state of allospecific versus TNP-self-specific pCTL. These investigators observed that pretreatment of T cell populations with anti-Lyt-1 plus complement eliminated anti-TNP-self CTL responses, but did not eliminate anti-allo-CTL responses. However, since anti-Lyt-1 plus complement treatment probably preferentially eliminates class II-restricted (i.e. L3T4⁺) T cells, our study indicates that the differences between the anti-allo- and anti-TNP-CTL responses that they observed (29) likely resulted from differences in the Lyt phenotypes of the Th cells required to initiate these two responses, in that anti-TNP responses strictly require class II-restricted L3T4⁺ Th cells, but anti-allo responses do not.

In conclusion, this study documents the participation of two distinct Th cell populations in the generation of class I allospecific CTL responses that can be distinguished by their Lyt phenotype as well as by their MHC restriction specificity. The specificity and biological significance of the Lyt-2⁺ Th cell population in immune responses will be the subject of further studies.

Summary

This study characterizes the T helper (Th) cells that initiate primary cytotoxic T lymphocyte (CTL) responses against allogeneic and trinitrophenyl (TNP)-modified self class I major histocompatibility (MHC) determinants. We show that two distinct Th cell subsets participate in allospecific CTL responses: (a) an L3T4⁺,Lyt-2⁻ class II-restricted Th cell population, and (b) an L3T4⁻,Lyt-2⁺ class I-restricted Th cell population. Both of these T cell subpopulations were shown to function in allospecific CTL responses as helper cells by their ability to show synergy with allospecific CTL precursors. Thus, primary class I allospecific CTL responses represent an immune response involving not only L3T4⁺ Th cells, but Lyt-2⁺ Th cells as well. One of the necessary functions performed by both L3T4⁺ and Lyt-2⁺ Th cell populations in allospecific CTL responses was found to be the secretion of interleukin 2. Finally, despite the many similarities between anti-allo- and anti-TNP-CTL responses, anti-TNP-CTL responses were found to be mediated by only L3T4⁺ Th cells, not by Lyt-2⁺ Th cells. Consequently, Lyt-2⁺ Th cells appear to be a helper cell population that is primarily involved in MHC-specific immune responses.

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