PROPERTIES OF PURIFIED T CELL SUBSETS

I. In Vitro Responses to Class I vs. Class II H-2 Alloantigens

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Monoclonal antibodies (mAb)[1] to cell surface L3T4 and Lyt-2 molecules define two broad classes of T cells (1, 2); both T cell subsets are restricted by H-2 molecules (3). $L3T4^+$ $Lyt\text{-}2^-$ T cells provide T helper (Th) function and are restricted by H-2 class II (I-A, I-E) (Ia) molecules, both in terms of H-2 alloreactivity and recognition of non H-2 antigens in the context of self H-2 molecules. $L3T4^-$ $Lyt\text{-}2^+$ T cells exert cell-mediated lympholysis (CML) and are restricted by H-2 class I (H-2K, H-2D) molecules. Whereas class II–restricted Th cells function independently of other T cells, it has been long argued that the function of cytotoxic lymphocytes (CTL) and their precursors requires interaction with Th cells (4–6). Although the mechanism of such T-T interaction is still debated, the simplest view is that Th cells facilitate CTL differentiation through the production of interleukin 2 (IL-2), a lymphokine known to control the growth of CTL (7). A key question is whether Th cells play an obligatory role in controlling CTL differentiation or merely augment the growth of these cells after the induction phase.

Recent evidence (8–10) suggests that, under certain circumstances, CTL or their precursors can differentiate without the requirement for exogenous help. Perhaps the first evidence supporting this notion came from the finding that depleting unprimed lymph node (LN) cells of Ia-restricted $Lyt\text{-}1^+$ T cells failed to impair lethal graft-vs-host disease (GVHD) directed to minor histocompatibility antigens in heavily irradiated mice (11, 12); purified $Lyt\text{-}1^-$ $Lyt\text{-}2^+$ T cells were as effective as unseparated T cells at causing GVHD, implying that help from $Lyt\text{-}2^-$ T cells was not required in this situation. Subsequently, several groups have reported the existence of $Lyt\text{-}2^+$ T cell clones that proliferate in response to antigen in vitro in the absence of exogenous IL-2 from $Lyt\text{-}2^-$ T cells (8–10).

The notion that, under certain conditions, $Lyt\text{-}2^+$ T cells can respond to antigen without the requirement for interaction with $Lyt\text{-}2^-$ T cells implies that purified $Lyt\text{-}2^+$ T cells should be capable of mounting primary mixed lymphocyte reaction (MLR) and CTL responses to alloantigens, especially to class I alloanti-

This work was supported by grants AI 21487, CA 38355, CA 25803, and CA 35048 from the U. S. Public Health Service.

[1] *Abbreviations used in this paper:* C′, complement; CML, cell-mediated lympholysis; CTL, cytotoxic lymphocytes; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GVHD, graft-vs.-host disease; HBSS, Hanks' balanced salt solution; HD, helper dependent; HI, helper independent; IL-2, interleukin-2; mAb, monoclonal antibody; LN, lymph node; MLR, mixed lymphocyte reaction; NMS, normal mouse serum; PBS, phosphate-buffered saline; rIL-2, recombinant IL-2; SN, supernatant; Th, T helper.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/85/12/2068/21 $1.00
Volume 162 December 1985 2068–2088

gens. In this respect it has long been held that primary MLR with unseparated T cells are lower to class I differences than to class II differences (13–15). A notable exception is that C57BL/6 (B6) T cells elicit high MLR to cells from strains such as B6.C-H-2^{bm1} (bm1) (15, 16), which are identical to B6 except for slight (two to three amino acids) differences in the H-2K molecule (17). Recently (18, 19), it has been reported that the response of B6 T cells to bm1 involves recognition of K^{bm1} molecules in the context of self class II molecules, implying that the response of class I–specific CTL precursor cells to bm1 is under the control of class II–specific Th cells. Others have contested this conclusion and argue that B6 T cells highly enriched for Lyt-2^+ cells respond well to bm1 (10). For rats, some workers have observed high primary MLR with purified OX-8^+ cells (20) (OX-8 is the homologue of Lyt-2). Others contend that the response of OX-8^+ cells in MLR reflects contamination with W2/25 Th cells (21).

The main aim of the present work was to prepare highly purified populations of L3T4^+ and Lyt-2^+ cells and determine which of these subsets participate in primary responses to allo class I vs. class II differences in vitro. In the case of Lyt-2^+ cells, particular emphasis was placed on using T cell proliferation (MLR) rather than CTL generation to quantitate the responses; MLR were demonstrable as early as day 2, well before CTL were detected. The results suggest that both T cell subsets function independently, L3T4^+ T cells accounting for anti–class II responses and Lyt-2^+ T cells for class I responses. In particular, no evidence was found that the response of Lyt-2^+ T cells to class I differences depended upon the presence of L3T4^+ T cells.

Materials and Methods

Mice. C57BL/6Kh (B6), C57BL/10 (B10), B6.C-H-2^{bm1} (bm1), B6.C-H-2^{bm4} (bm4), B6.C-H-2^{bm9} (bm9), B6.C-H-2^{bm11} (bm11), B6.C-H-2^{bm12} (bm12), B10.BR, B10.A, B10.A(4R), B10.A(2R), B10.P, and CBA/Ca (CBA) mice were obtained from the breeding colony of the Research Institute of Scripps Clinic. B10.AQR and B10.TL mice were kindly provided by Dr. Chella David (Mayo Clinic).

Irradiation. Cells were exposed to 1,500 rad of irradiation from a ^{137}Cs source (500 rad/min) delivered by a Gamma cell 1000 irradiator (Atomic Energy of Canada, Ottawa, Canada).

Media. RPMI 1640 and Hanks' balanced salt solution (HBSS) were used. For cell separation and purification, medium (usually HBSS) was supplemented with 5% γ-globulin-depleted horse serum (Gibco Laboratories, Santa Clara, CA) and Hepes. For MLR and CTL generation, RPMI 1640 was supplemented with 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA) or 0.5% fresh normal mouse serum (NMS), 5% NCTC 109, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics.

Monoclonal Antibodies. The following mAb were used: hybridoma GK1.5 (anti-L3T4, rat IgG2b), ascites fluid (diluted 1:10 as stock solution) (2); hybridoma 3.168.8 (anti-Lyt-2, rat IgM), ascites fluid (22); hybridoma J1j (anti-Thy 1.2, rat IgM), ascites fluid (diluted 1:10 as stock solution) (23); hybridoma 11-5.2 (anti-I-A^k, mouse IgG2b), ascites fluid (24); hybridoma BP107 (anti-I-A^b, mouse IgG2a), ascites fluid (25); hybridoma J11d (lytic for B cells but not mature T cells, rat IgM), culture supernatant (23); and hybridoma 20-8-4s (anti-K^bD^b, mouse IgG2a), protein A–purified ascites fluid (26). With the exception of J11d, all hybridomas were grown in ascites form in (B6 × CBA)F_1 mice preconditioned with Pristane (0.5 ml intraperitoneally 1–2 wk before), antilymphocyte serum (M. A. Bioproducts, Walkersville, MD) (50–100 μl given 2–3 d before) and whole body irradiation (500–600 rad 4–6 h before). Cytotoxic titers of ascites fluid were in the range of 5×10^4 to 5×10^5. For J11d, culture supernatant from cells grown in vitro was used. Guinea pig

serum absorbed with mouse spleen cells was used as a source of complement (C'). Guinea pig serum obtained from commercial sources gave erratic results with several of the hybridomas, especially GK1.5; preparation of our own guinea pig serum generally gave the best results.

Purification of Responder Cells for MLR. Cell suspensions of pooled axillary, inguinal, cervical and mesenteric LN were prepared with tissue homogenizers. Semipurified populations of Lyt-2$^+$ and L3T4$^+$ cells were made in a one-step procedure by treating LN cells with a mixture of J11d plus anti-L3T4 mAb plus C' (for Lyt-2$^+$ cells) or J11d plus anti-Lyt-2 mAb plus C' (for L3T4$^+$ cells). The concentrations of mAb used to treat the LN cells were: J11d, 0.3 ml of undiluted culture supernatant per 5×10^7 cells; anti-L3T4, 0.1 ml of ascites fluid (prediluted 1:10) per 5×10^7 cells; anti-Lyt-2, 0.1 ml of ascites fluid per 5×10^7 cells. Cells were incubated at a final concentration of 5×10^7 cells/ml for 60 min at 37°C and washed three times before further use.

Further purification of T cell subsets was achieved by panning. To prepare purified Lyt-2$^+$ cells, semipurified cells (LN pretreated with J11d, anti-L3T4, and C') were placed on 100 × 15 mm petri dishes coated with anti-Lyt-2$^+$ mAb. Dishes were coated with 5 ml of a 1:50 dilution of mAb in phosphate-buffered saline (PBS) for 1 h at room temperature. After pouring off the mAb solution, the plates were rinsed five times with PBS and once with HBSS plus 5% horse serum. Cell doses in the range of 10^7 to 2.5×10^7 viable cells per plate were poured onto the plates in a volume of 3.5 ml of HBSS plus 5% horse serum. Cells were incubated on the dishes for 1 h at 4°C. Nonadherent cells were removed by very carefully washing the plates five times with medium. Adherent cells were eluted from the dishes by vigorous pipetting with a Pasteur pipette. Fluorescence-activated cell sorter (FACS) analysis (see Table I) indicated that the eluted cells were not coated with antibody.

Percoll Separation. To attempt to deplete responder cells of IL-2-responsive cells, dense cells were harvested from band 5 (1.09 density interface) of Percoll gradients prepared according to the method of Ratcliffe and Julius (27).

Cell Typing. Antibody-mediated lysis in the presence of C' was measured in a one-step assay as described elsewhere (23). Cell viability was assessed by phase contrast microscopy. To stain cells for FACS analysis, aliquots of 10^6 cells in 100 μl of HBSS supplemented with 5% horse serum and 0.1% sodium azide were incubated at 4°C for 30 min with 25 μl of mAb (undiluted ascites fluid), washed three times, and then incubated for a further 30 min at 4°C with 5 μg fluorescein isothiocyanate (FITC)-labeled mouse anti-rat IgG (heavy and light chain specific) (Pel-Freez Biologicals, Rogers, AR). After being washed three times, the labeled cells were analysed on a FACS IV flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Purification of Stimulator Cells for MLR. Spleen cells were depleted of T cells by incubation with anti-Thy-1 mAb (0.1 ml of J1j ascites fluid diluted 1:10 per 5×10^7 cells) plus C'. To deplete bm1 spleen cells of Ia$^+$ cells, cells were cultured with anti-I-A^b mAb (0.05 ml of BP107 ascites fluid per 1.5×10^7 cells) plus C'. In both situations the cells were incubated with mAb plus C' for 60 min, washed thoroughly, exposed to 1,500 rad, and then used as stimulator cells.

MLR. Unless stated otherwise, $1-2 \times 10^5$ responder cells were cultured in flat-bottom microtiter plates with 5×10^5 irradiated (1,500 rad) spleen cells as stimulators in a volume of 200 μl. Cultures were pulsed with 1 μCi [^{3}H]thymidine ([^{3}H]TdR) and harvested 18 h later.

Generation of CTL. 2×10^6 B6 T cells or B6 Lyt-2$^+$ cells were cultured with 5×10^6 1,500 rad bm1 spleen cells in a volume of 2 ml in 24-well plates. After 4 d the cells pooled from several wells were washed and counted (only blast cells were counted); the number of cells recovered (>90% blasts) were equivalent to, or exceeded, the number of responders initially plated, cell recoveries being appreciably higher with B6 Lyt-2$^+$ than B6 T cells. To measure CTL activity, varying numbers of responder cells were cultured for 3 h at 37°C with fixed numbers (10^4) of ^{51}Cr-labeled target cells (spleen cells cultured for 3 d with concanavalin A, 5 μg/ml); target cells were labeled with 300 μCi ^{51}Cr per 4×10^6 cells at 37°C for 1 h and then washed three times. The percent ^{51}Cr release from

target cells was measured by standard techniques, taking release of isotope from detergent-treated cells as 100% release.

Negative Selection. LN cells were filtered from blood to thoracic duct lymph through irradiated H-2-different mice as described elsewhere (28).

IL-2. Recombinant human IL-2 (rIL-2) was kindly provided by Cetus Corp., Emeryville, CA. Supernatant of rat lymphoid cells stimulated in vitro with concanavalin A (Con A SN) was generously made available by Dr. S. Webb of this institution; the batch of Con A SN used had a high titer in terms of promoting growth of IL-2-dependent HT-2 cells.

Results

In testing the function of the two subsets of T cells in MLR, it was considered essential to use highly purified populations of cells as responders. The approach used for cell purification is outlined below.

Preparation of Purified T Cell Subsets. Treatment of normal LN cells with J11d (anti–B cell) mAb plus C′ yielded a population containing 98–100% of Thy-1$^+$ cells (Table I, group A); J11d has no detectable reactivity for peripheral T cells (23). When tested by cytotoxicity or by FITC staining, the ratio of L3T4$^+$ to Lyt-2$^+$ T cells in J11d-treated LN ranged from 1:1 (Table I, group A; Fig. 1) to 2:1, depending upon the strain and age of the cell donors. When LN cells were treated with a mixture of J11d, anti-L3T4 mAb, and C′, 85–95% of the surviving cells were positive for Lyt-2 (Table I, group B); virtually none of the cells were positive for L3T4, even by FITC staining. Cells treated with J11d, anti-L3T4 mAb and C′ were further purified by panning, i.e., by placing the cells on anti-Lyt-2–coated plastic dishes for 1 h at 4°C, washing away the nonadherent cells, and then eluting the adherent cells by vigorous pipetting (Materials and Methods). As assessed by both cytotoxicity and FITC staining, the cells obtained by this procedure were >99% Lyt-2$^+$ and contained no detectable L3T4$^+$ cells (Table I, group D; Fig 1). Cytotoxic testing of five consecutive batches of these cells gave mean cytotoxic indices of 99% with anti-Lyt-2 mAb and <1% with anti-L3T4 mAb. An analogous procedure was used to prepare L3T4$^+$ cells. LN cells surviving treatment with J11d, anti-Lyt-2 mAb, and C′ contained 85–95% L3T4$^+$ cells and no detectable Lyt-2$^+$ cells (Table I, group C). Panning on anti-L3T4–coated dishes yielded a population containing ~100% L3T4$^+$ cells with no detectable Lyt-2$^+$ cells (Table I, group E; Fig. 1).

MLR to Full H-2 Differences. An initial experiment with B10 T cells as responders is shown in Table II, Exp. 1. MLR were measured on day 3. As expected, purified B10 (*H-2^b*) T cells (cells treated with J11d only) responded well to B10.BR (*H-2^k*) stimulators and gave relatively low background counts with syngeneic stimulators. When semipurified L3T4$^+$ cells (cells treated with J11d, anti-Lyt-2 mAb, and C′ but not panned) were used as responders, the response to B10.BR decreased. With semipurified Lyt-2$^+$ cells as responders, by contrast, the response to B10.BR increased.

MLR to bm1 vs. bm12 Stimulators. Similar cell populations derived from B6 LN were tested for their capacity to respond to bm1 vs. bm12 stimulators, i.e., stimulators differing only at class I (bm1) or class II (bm12) loci (Table II, Exp. 2). Whereas B6 T cells responded well to both bm1 and bm12 stimulators, semipurified L3T4$^+$ cells showed an increased response to bm12 but gave virtually no response to bm1. The reverse applied to semipurified Lyt-2$^+$ cells.

TABLE I

Surface Markers of Semipurified vs. Highly Purified Lyt-2$^+$ and L3T4$^+$ LN Cells

Group	Pretreatment of B6 LN*	Assay for typing cells	Percent of cells reactive with mAb: Anti-Thy-1.2	Anti-L3T4	Anti-Lyt-2
A	J11d + C′	C′-mediated cytotoxicity‡	99	45	51
		FITC staining§	98.5	47.4	50.1
B	J11d + anti-L3T4 + C′	C′-mediated cytotoxicity	98	0	89
		FITC staining	98.0	−0.7	93.5
C	J11d + anti-Lyt-2 + C′	C′-mediated cytotoxicity	98	88	0
		FITC staining	96.6	92.7	−0.4
D	J11d + anti-L3T4 + C′, then panned on anti-Lyt-2–coated plates	C′-mediated cytotoxicity	100	0	100
		FITC staining	99.8	−0.2	99.4
E	J11d + anti-Lyt-2 + C′, then panned on anti-L3T4–coated plates	C′-mediated cytotoxicity	100	99	0
		FITC staining	99.7	99.8	−0.1

* Normal B6 LN cells were treated in the presence of C′ with J11d mAb (group A), J11d + anti-L3T4 mAb (group B) or J11d + anti-Lyt-2 mAb (group C) for 60 min at 37°C (see Materials and Methods for details). After washing, aliquots of the group B and group C cells were placed on plastic dishes coated with anti-Lyt-2 (group D) or anti-L3T4 mAb (group E) for 60 min at 4°C. After gentle rinsing with medium to remove nonadherent cells, the adherent cells were eluted from the dishes by vigorous pipetting.

‡ Dead cells were first removed by Ficoll separation. The data are shown in terms of cytotoxic indices; background lysis with cells treated with C′ alone varied from 1 to 8%. Viability was assessed on >600 cells per sample.

§ Cells were typed by incubating cells with anti-Thy-1, anti-L3T4, or anti-Lyt-2 mAb followed by an FITC anti–rat Ig antiserum (Materials and Methods). Labeled cells were detected by FACS analysis. Background labeling observed with FITC anti–rat Ig alone was 1.1% for group A, 7.5% for group B, 3.0% for group C, 1.7% for group D, and 1.4% for group E. The data are shown in terms of a staining index, i.e., [(percent staining experimental group − percent staining with FITC anti–rat Ig) ÷ (100 − percent staining with FITC anti–rat Ig)] × 100.

Here there was a high response to bm1 but a low response to bm12. Exp. 3 of Table II shows the effects of using highly purified ("kill pan") B6 Lyt-2$^+$ T cells as responders. Three points are evident. First, the background counts with syngeneic stimulators were almost nonexistent. Second, the responses to bm1 were conspicuously high. Third, in contrast to the use of semipurified Lyt-2$^+$ cells (Exp. 2), there was virtually no response to bm12. F_1 stimulators were used in these experiments to minimize backstimulation (29). To further minimize backstimulation, the stimulators (F_1 or homozygous) in several subsequent experiments (e.g., Tables III and IV and Figs. 2 and 3) were pretreated with anti-Thy-1 mAb plus C′ to remove T cells, a potential source of IL-2. In no case did the use of anti-Thy-1–treated stimulators lower the response of purified Lyt-2$^+$ cells. In all of the experiments considered below, purified Lyt-2$^+$ and L3T4$^+$ cells were prepared by the combination of killing with the reciprocal mAb followed by positive panning. For simplicity these purified cell populations will

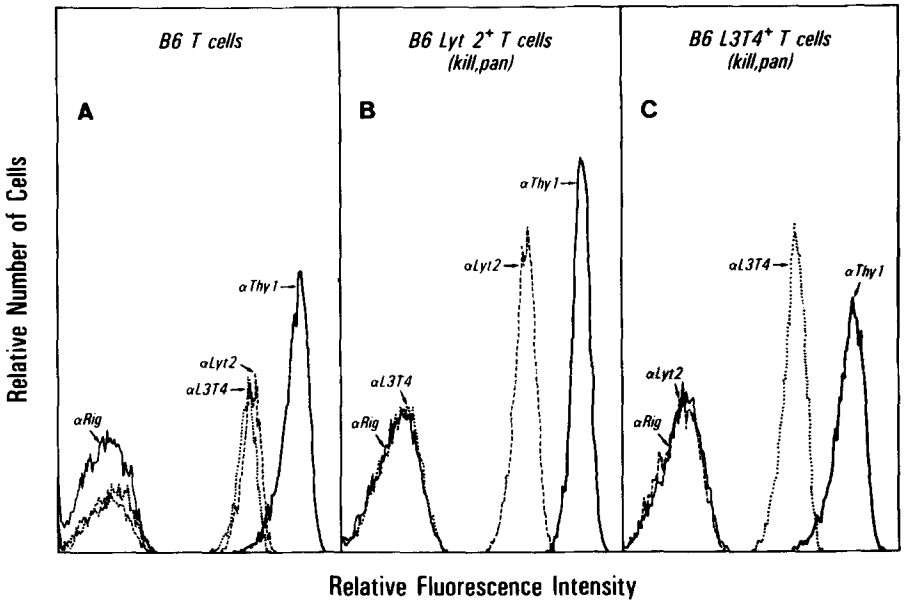

FIGURE 1. FACS profile of purified B6 LN T cells (*A*), Lyt-2$^+$ cells (*B*), and L3T4$^+$ cells (*C*) incubated with anti-L3T4, -Lyt-2, or -Thy-1.2 mAb followed by FITC-labeled anti-rat Ig (*αRig*). See Table I footnotes and Materials and Methods for details.

TABLE II

MLR by Semipurified vs. Highly Purified Lyt-2$^+$ T Cells: Response to bm1 vs. bm12

Exp. No.	Responders	Pretreatment of responders	No. of responders	[^{3}H]TdR ($\times 10^{-3}$) incorporation with stimulators (5×10^5)*		
				B10	B10.BR	
1	B10 T	J11d + C′	2×10^5	1.3 (0.5)	54.8 (0.8)	
	B10 L3T4$^+$	Anti-Lyt-2 + C′	2×10^5	2.4 (0.2)	16.8 (3.6)	
	B10 Lyt-2$^+$	Anti-L3T4 + C′	2×10^5	0.9 (0.5)	73.0 (3.5)	
				B6	bm1	bm12
2	B6 T	J11d + C′	1×10^5	1.5 (0.5)	40.3 (6.2)	16.0 (2.5)
	B6 L3T4$^+$	Anti-Lyt-2 + C′	1×10^5	4.1 (0.6)	4.6 (0.6)	26.8 (2.6)
	B6 Lyt-2$^+$	Anti-L3T4 + C′	1×10^5	0.7 (0.0)	79.4 (4.8)	4.4 (1.3)
				B6	(B6 × bm1)F_1	(B6 × bm12)F_1
3	B6 Lyt-2$^+$	Anti-L3T4 + C′, then panned on anti-Lyt-2–coated plates	1×10^5	0.2 (0.1)	53.0 (5.5)	0.7 (0.3)
			2×10^5	0.7 (0.3)	105.8 (2.5)	0.9 (0.2)

* Data are shown as the mean of triplicate cultures; SD are shown in parentheses. [^{3}H]TdR incorporation measured on day 3 of culture. Cells cultured in 10% FCS.

be referred to as Lyt-2$^+$ and L3T4$^+$ cells. It will be noted below that the background counts observed when Lyt-2$^+$ cells were cultured with syngeneic stimulators were almost undetectable. With L3T4$^+$ cells, by contrast, background counts (auto-MLR) were often very high, particularly when cells were cultured in FCS. Many experiments with L3T4$^+$ cells had to be discarded because of high background counts, and only the optimal experiments are presented.

A time course of the response of B6 Lyt-2$^+$ and L3T4$^+$ cells in MLR is shown in Fig. 2; cells were cultured either in 0.5% NMS (Fig. 2, *top*) or 10% FCS (Fig. 2, *bottom*). In the case of B6 L3T4$^+$ cells, it can be seen that the response to bm12 rose progressively and reached maximum values on day 6; a similar time course was observed with responses to (B6 × CBA/Ca)F_1 stimulators, i.e., to a full H-2 difference. Responses of L3T4$^+$ cells to bm1 were almost nonexistent.

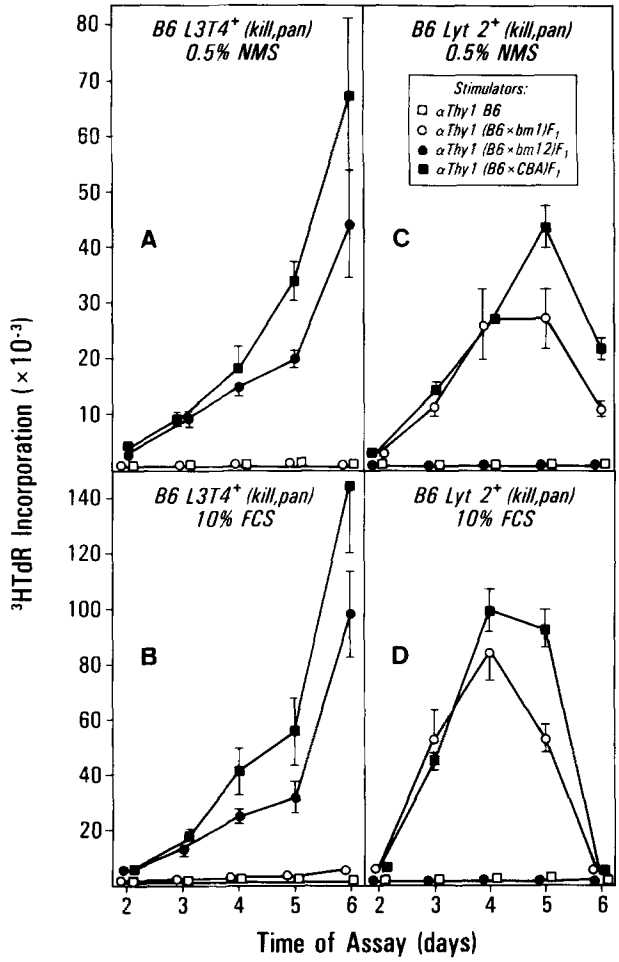

FIGURE 2. Time course of MLR of purified B6 LN L3T4$^+$ and Lyt-2$^+$ cells stimulated against class I (bm1) vs. class II (bm12) differences or against a whole H-2 difference (CBA, H-2^k); 1 × 10^5 responder cells. Cells were cultured in either 10% FCS or 0.5% NMS. Stimulators were pretreated with anti-Thy-1 mAb + C′.

With B6 Lyt-2$^+$ responders, the responses to bm1 and (B6 × CBA)F_1 reached peak values on day 4 (FCS) or day 4–5 (NMS) and then declined precipitously. Responses to bm12 were virtually undetectable.

MLR to Other Mutant Class I Differences. As shown in Table III, B6 Lyt-2$^+$ cells gave high responses not only to bm1 but also to bm4 and bm11. Much lower responses were detected against bm9. High responses were observed against various full H-2 differences, including B10.BR (Table III), B10.P (*H-2^p*) and B10.D2 (*H-2^d*) (data not shown).

MLR to Allelic Class I Differences. In Exp. 1 of Table IV, B10.A T cells and B10.A Lyt-2$^+$ cells were compared for their capacity to respond to various H-2K/D allelic differences (D^k, D^b, K^q, K^s). It is evident that B10.A Lyt-2$^+$ cells gave approximately twofold higher responses to these differences than B10.A T cells in three of the four combinations tested.

Exp. 2 of Table IV demonstrates that B10.A(4R) Lyt-2$^+$ cells failed to respond to an allelic class II difference, i.e., to I-E^k molecules expressed by B10.A(2R) cells. This finding paralled the unresponsiveness of B6 Lyt-2$^+$ cells to the class II mutant, bm12 (see above).

MLR to Class I Differences Across an Ia Barrier. The finding that Lyt-2$^+$ T cells responded well to a full H-2 difference yet failed to respond to stimulators differing only at class II loci (Tables III, IV) implied that the response of Lyt-2$^+$ T cells to class I differences did not require Ia compatibility with the stimulators. The experiment shown in Exp. 3 of Table IV provides direct support for this notion. Here it can be seen that CBA (Ia^k) Lyt-2$^+$ cells negatively selected against B6 (Ia^b) H-2 determinants in vivo gave no response to B6 in vitro but responded well to bm1. (See Table IV footnote for details of the procedure for negative selection.)

MLR in the Presence of Added IL-2. The effects of adding rIL-2 (100 U/ml) to MLR elicited by Lyt-2$^+$ and L3T4$^+$ cells is shown in Fig. 3. B6 L3T4$^+$ and B6 Lyt-2$^+$ cells were stimulated against bm12 and bm1 cells, respectively; for convenience the data are plotted on a log scale. In the case of L3T4$^+$ cells, it is evident the response to bm12 stimulators increased exponentially from days 2 to 6. Addition of IL-2 had no effect on this response. The MLR of Lyt-2$^+$ cells

TABLE III

Response of Purified Lyt-2$^+$ T Cells to Various Mutant Class I H-2 Differences

Responders	Stimulators (anti-Thy-1–treated)	*H-2* haplotype of stimulators*	[^{3}H]TdR incorporation ($\times 10^{-3}$)‡	
			No. of responders:	
			1×10^5	2×10^5
B6 Lyt-2$^+$	B6	*bbbb*	0.2 (0.0)‡	0.3 (0.1)
	bm1	*bm1bbb*	155.3 (23.11)	336.5 (16.2)
	bm4	*bm4bbb*	57.3 (8.4)	120.3 (12.3)
	bm9	*bm9bbb*	11.8 (1.1)	33.5 (2.5)
	bm11	*bm11bbb*	179.3 (4.7)	275.8 (28.3)
	bm12	*bbm12bb*	0.4 (0.1)	2.6 (1.8)
	B10.BR	*kkkk*	79.3 (14.7)	257.4 (22.3)

* *K, I-A, I-E, D.*

‡ Mean of triplicate cultures (SD). Responses measured on day 4; cells cultured in 10% FCS.

TABLE IV

Response of Purified Lyt-2$^+$ T cells to Various Allelic Class I H-2 Differences

Exp. No.	Responders (2×10^5)	Stimulators (anti-Thy-1–treated)	*H-2* haplotype of stimulators*	Stimulus	[^{3}H]TdR incorporation ($\times 10^{-3}$) with stimulators‡
1	B10.A T	B10.A	*kkkd*	None	1.1 (0.2)
		B6	*bbbb*	H-2^b	86.6 (5.1)
		B10.BR	*kkkk*	D^k	28.6 (2.0)
		B10.A(4R)	*kkbb*	D^b	12.9 (1.7)
		B10.AQR	*qkkd*	K^q	24.4 (3.0)
		B10.TL	*skkd*	K^s	16.5 (3.7)
	B10.A Lyt-2$^+$	B10.A	*kkkd*	None	0.3 (0.1)
		B6	*bbbb*	H-2^b	72.8 (14.3)
		B10.BR	*kkkk*	D^k	60.3 (12.4)
		B10.A(4R)	*kkbb*	D^b	23.9 (3.4)
		B10.AQR	*qkkd*	K^q	31.1 (6.4)
		B10.TL	*skkd*	K^s	29.6 (1.4)
2	B10.A(4R) Lyt-2$^+$§	B10.A(4R)	*kkbb*	None	0.2 (0.0)
		B6	*bbbb*	K^b, I-A^b	21.2 (0.4)
		B10.BR	*kkkk*	I-E^k, D^k	19.5 (1.0)
		B10.A(2R)	*kkkb*	I-E^k	0.6 (0.2)
3	CBA Lyt-2$^+$ filtered through 900 rad B6‖	B6	*bbbb*	(H-2^b)	0.2 (0.0)
		bm1	*bm1bbb*	(H-2^b), K^{bm1}	48.7 (4.4)
		B10.P	*pppp*	H-2^p	38.2 (7.7)

* *K, I-A, I-E, D.*

‡ Responses were measured on day 4; cells cultured in 10% FCS (Exp. 1, 3) or 0.5% NMS (Exp. 2).

§ Unseparated B10.A(4R) T cells responded well to B10.A(2R) stimulators.

‖ Normal CBA LN cells were pretreated with J11d + anti-L3T4 mAb + C′ and then transferred intravenously into B6 mice given 900 rad irradiation 4 h before. Cells were transferred into three mice, each mouse receiving 8×10^7 viable lymphocytes. Thoracic duct cannulation (28) was performed 16 h later and lymph-borne cells were collected between 18 and 42 h after injection; cells collected during this period are unresponsive to host alloantigens and are almost entirely of donor origin (28). The lymph-borne cells were panned on anti-Lyt-2–coated dishes (Materials and Methods); purified Lyt-2$^+$ cells eluted from the dishes were then used as responders in MLR.

to bm1 also increased exponentially, but only until day 4; thereafter the response dropped sharply. Addition of IL-2 to cultures of Lyt-2$^+$ cells increased the anti-bm1 responses observed between days 2 and 4 by about twofold. This mild increase in the B6 Lyt-2$^+$ anti-bm1 response caused by IL-2 was offset by a dramatic increase in the background response with syngeneic stimulators; this applied despite prior separation of the responders on Percoll gradients to select for dense cells.

MLR in the Presence of Anti-L3T4 and Anti-Lyt-2 mAb. Although the Lyt-2$^+$ cells used in the above experiments contained no detectable L3T4$^+$ cells, the possibility that the response of the Lyt-2$^+$ cells depended on minimal (<0.5%) contamination with L3T4$^+$ cells could not be excluded. Evidence against this possibility is shown in Table V. Here it can be seen that the MLR of B6 Lyt-2$^+$ cells to bm1 stimulators was not affected by addition of anti-L3T4 mAb to the cultures; by contrast, adding anti-Lyt-2 mAb abolished the response to bm1. In

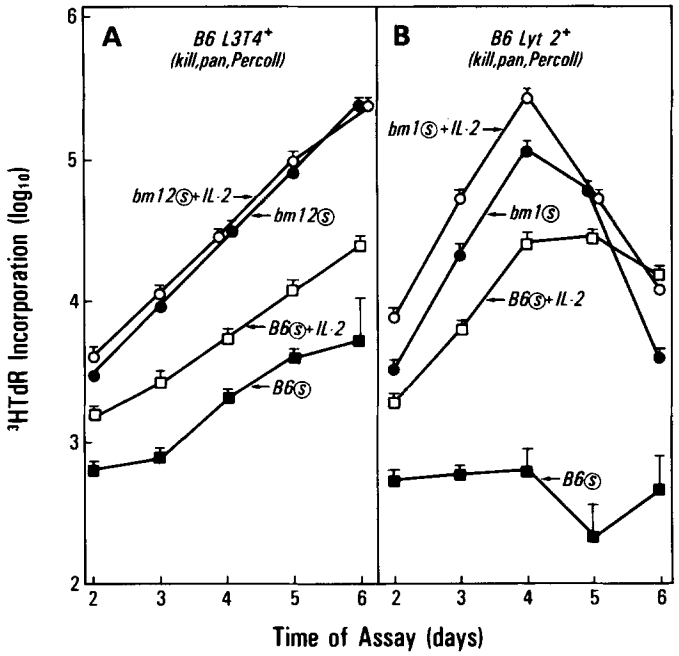

FIGURE 3. Effect of adding rIL-2 (final concentration, 100 U/ml) to MLR of B6 L3T4$^+$ cells responding to a class II difference (bm12) (*A*) vs. B6 Lyt-2$^+$ cells responding to a class I difference (bm1) (*B*). All stimulator (Ⓢ) cells (5×10^5 viable cells per well) were pretreated with anti-Thy-1 mAb + C′. Responder cells were first placed on Percoll gradients; cells removed from the band 5 fraction (enriched for dense cells) were used as responders at a dose of 10^5 cells/well. Mean of triplicate cultures; cells cultured in 10% FCS.

the case of L3T4$^+$ responders, MLR to bm12 stimulators were abolished by anti-L3T4 mAb but unaffected by anti-Lyt-2 mAb.

An objection to the use of purified Lyt-2$^+$ cells in the above experiments is that the procedures used to separate the cells might have provided an unphysiological signal that enabled the cells to respond to antigen in the absence of L3T4$^+$ cells. If so, anti-L3T4 mAb would be expected to block the response of unseparated T cells to class I differences. This was not found to be the case. As shown in Table VI, the response of normal LN cells or LN T cells to bm1 measured on day 3 was only marginally inhibited by anti-L3T4 mAb; responses to bm12, by contrast, were virtually abolished by anti-L3T4 mAb. Reciprocal blockage was observed with anti-Lyt-2 mAb. It may be noted that the response of LN or LN T cells to a whole H-2 difference (B10.BR) was inhibited more effectively by anti-Lyt-2 than by anti-L3T4 mAb. This finding is in accordance with the observation that day 3–4 MLR to whole H-2 differences were generally higher with purified Lyt-2$^+$ than L3T4$^+$ cells (Fig. 2; see also Table II, Exp. 1).

Antigen-presenting Cells for Lyt-2$^+$ Cells. The finding that Lyt-2$^+$ cells were not inhibited by anti-L3T4 mAb and responded to class I molecules across Ia barriers suggested that recognition of allo class I molecules by Lyt-2$^+$ cells did not involve co-recognition of self Ia molecules. Significantly, however, pretreatment of stimulator cells with anti-Ia mAb plus C′ abolished the response of Lyt-

TABLE V

Capacity of Anti-L3T4 vs. Anti-Lyt-2 mAb to Inhibit MLR to Class I vs. Class II H-2 Differences: Purified T Cell Subsets as Responders

Responders (1×10^5)	mAb added to MLR*		[^{3}H]TdR incorporation ($\times 10^{-3}$) in response to:‡		
	Anti-L3T4	Anti-Lyt-2	B6	bm12	bm1
	%				
B6 L3T4$^+$	0	0	2.5	15.1	1.6
	1	0	0.4	1.5	—
	5	0	—	1.3	—
	0	1	2.2	15.8	—
	0	5	—	17.4	—
B6 Lyt-2$^+$	0	0	0.4	2.9	49.3
	1	0	0.5	0.9	54.3
	5	0	—	0.7	50.4
	0	1	0.4	0.4	1.2
	0	5	—	0.4	0.8

* 200-μl cultures supplemented with 2 μl or 10 μl of undiluted ascites fluid (anti-Lyt-2) or ascites fluid previously diluted 1:10 (anti-L3T4).
‡ Mean of triplicate cultures; for simplicity, SD have been omitted. Cells were cultured in 10% FCS; MLR measured on day 3.

TABLE VI

Capacity of Anti-L3T4 vs. Anti-Lyt-2 mAb to Inhibit MLR to Class I vs. Class II H-2 Differences: Unseparated LN Cells as Responders

Responders* (2×10^5)	mAb added to MLR‡		[^{3}H]TdR incorporation ($\times 10^{-3}$) in response to:§			
	Anti-L3T4	Anti-Lyt-2	B6	bm1	bm12	B10.BR
	%					
Unseparated B6 LN	0	0	5.3	59.6	26.6	64.4
	1	0	2.5	50.8	5.3	44.7
	0	1	4.4	6.8	24.9	18.4
B6 LN T	0	0	3.6	69.1	28.4	74.8
	1	0	1.1	64.3	4.4	54.3
	0	1	3.4	6.7	26.6	19.7

* Responders were either untreated LN cells or LN cells pretreated with J11d mAb + C′ to remove B cells.
‡ See Table V.
§ As for Table V.

2$^+$ cells. This is exemplified by two experiments shown in Table VII. It can be seen that the response of B6 Lyt-2$^+$ cells to bm1 stimulators was reduced to near background levels by pretreating the stimulators with anti-I-A^b mAb plus C′; anti-Ia–treated stimulators were washed thoroughly before culture to prevent carry-over of antibody. Adding a source of syngeneic antigen-presenting cells (APC), i.e., up to 5×10^5 anti-Thy-1 plus C′–treated B6 spleen cells, had little or no effect in restoring the response. Only a slight (15–20%) restoration of the response occurred when the cultures were supplemented with 10% Con A SN

TABLE VII

Abolition of B6 Lyt-2$^+$ Anti-bm1 MLR by Pretreatment of Stimulators with Anti-I-A^b + C′: Effect of Adding Syngeneic Spleen Cells or IL-2

Exp. No.	Responders (1×10^5)*	Stimulators (pretreatment in presence of C′)‡	[^{3}H]TdR incorporation ($\times 10^{-3}$)
1	B6 Lyt-2$^+$	2.5×10^5 B6	0.1 (0.0)§
		5×10^5 B6	0.5 (0.5)
		2.5×10^5 bm1	16.7 (2.3)
		5×10^5 bm1	39.9 (3.0)
		2.5×10^5 bm1 (anti-I-A^b)	1.0 (0.7)
		5×10^5 bm1 (anti-I-A^b)	1.6 (0.5)
		2.5×10^5 bm1 (anti-I-A^b) + 2.5×10^6 B6	1.4 (0.4)
2	B6 Lyt-2$^+$	5×10^5 B6 (anti-Thy-1)	0.3 (0.2)
		1×10^6 B6 (anti-Thy-1)	0.4 (0.3)
		2.5×10^5 bm1 (anti-Thy-1)	9.9 (1.0)
		5×10^5 bm1 (anti-Thy-1)	19.4 (2.3)
		2.5×10^5 bm1 (anti-I-A^b)	0.7 (0.3)
		5×10^5 bm1 (anti-I-A^b)	0.9 (0.3)
		2.5×10^5 bm1 (anti-I-A^b) + 2.5×10^5 B6 (anti-Thy-1)	0.9 (0.6)
		2.5×10^5 bm1 (anti-I-A^b) + 5×10^5 B6 (anti-Thy-1)	1.1 (0.2)
		5×10^5 bm1 (anti-I-A^b) + 2.5×10^5 B6 (anti-Thy-1)	1.5 (0.4)
		5×10^5 bm1 (anti-I-A^b) + 5×10^5 B6 (anti-Thy-1)	2.1 (0.2)
		5×10^5 B6 (anti-Thy-1) + 10% Con A SN‖	3.6 (0.5)
		5×10^5 B6 (anti-Thy-1) + rIL-2¶	4.2 (0.3)
		5×10^5 bm1 (anti-Thy-1) + 10% Con A SN	34.8 (1.9)
		5×10^5 bm1 (anti-Thy-1) + rIL-2	33.7 (3.2)
		5×10^5 bm1 (anti-I-A^b) + 10% Con A SN	9.5 (0.7)
		5×10^5 bm1 (anti-I-A^b) + rIL-2	8.7 (0.3)

* Lyt-2$^+$ cells were placed on Percoll gradients; cells harvested from band 5 of the gradients were used as responders.

‡ Spleen cells were used to prepare stimulator cells; all stimulators were exposed to 1,500 rad before culture. In Exp. No. 2, (B6 × bm1)F$_1$ rather than homozygous bm1 cells were used as stimulators. Stimulators were pretreated with either anti-Thy-1 + C′ or anti-I-A^b + C′, and washed extensively before culture. Numbers of stimulator cells refer to numbers of viable cells.

§ Mean of triplicate cultures (SD); MLR in 10% FCS measured on day 3.

‖ Con A SN was supplemented with α-methylmannoside.

¶ rIL-2 was added at a final concentration of 100 U/ml.

or rIL-2 (100 U/ml) (Table VII); no further elevation in the response occurred when the dose of rIL-2 was increased 10-fold (data not shown).

Blocking Effects of Anti–Class II and Anti–Class I mAb. >10 experiments were performed to test whether addition of anti-Ia mAb to Lyt-2$^+$ cells in the absence of C′ caused inhibition of MLR to class I differences. The general trend in these

experiments was that anti-Ia antibodies directed to stimulator (but not responder) Ia determinants did cause a mild inhibition of the response to class I differences, but only if the control responses were relatively low. The most consistent results were seen when responses were measured in NMS rather than FCS. A representative experiment is shown in Table VIII. In this and other experiments three different concentrations of anti-Ia mAb were used to test for inhibition. For simplicity the data obtained with only one concentration are shown; higher concentrations of antibody often caused nonspecific inhibition.

It can be seen from Table VIII that anti-I-A^b but not anti-I-A^k mAb inhibited the response of B6 Lyt-2^+ cells to bm1 by ~30%. Conversely, anti-I-A^k but not anti-I-A^b mAb caused a 40% reduction in the response of B6 Lyt-2^+ cells to B10.A(4R) (I-A^k) stimulators. A similar mild degree of inhibition occurred when F_1 hybrid cells were used as responders, e.g., (B6 × CBA)F_1 cells, which express six Ia molecules (four I-A and two I-E molecules). (B6 and B10.A(4R) cells each express only one set of Ia (I-A) molecules.) In contrast to the modest (though specific) blocking effects of anti-Ia mAb on MLR involving Lyt-2^+ cells, the same concentrations of anti-I-A^b and anti-I-A^k mAb caused marked (80–90%) inhibition of the response of bm12 L3T4$^+$ cells to B6 and B10.A(4R) cells, respectively (Table VIII).

Antibodies to class I molecules were highly effective at inhibiting responses of Lyt-2^+ cells. Thus, as shown in Table VIII, Exp. 2, the response of bm1 Lyt-2^+ cells to B6 (K^bD^b) stimulators was almost totally abolished when anti-K^bD^b mAb was added to the cultures. This antibody failed to inhibit the response to K^k

TABLE VIII

Capacity of Anti–Class II and Anti–Class I mAb to Block MLR of L3T4$^+$ vs. Lyt-2$^+$ T Cells

Exp. No.	Responders (1×10^5)	mAb added to cultures*		[^{3}H]TdR incorporation ($\times 10^{-3}$) against stimulators (percent suppression):‡					
				B6	B10.A(4R)	(B6 × CBA)F_1	bm12	bm1	B10.BR
			Stimulus:	I-A^b	K^k, I-A^k	I-A^b, H-2^k	none	—	—
1	bm12 L3T4$^+$	—		30.5	18.3	56.1	0.8	—	—
		Anti-I-A^b		7.1 (79%)	16.4 (11%)	36.5 (33%)	—	—	—
		Anti-I-A^k		29.5 (3%)	4.0 (82%)	36.3 (33%)	—	—	—
		Anti-I-A^b + α I-A^k		—	—	21.2 (62%)	—	—	—
			Stimulus:	None	K^k, I-A^k	H-2^k	I-A^b	K^{bm1}	—
	B6 Lyt-2$^+$	—		0.1	13.2	13.1	0.3	26.1	—
		Anti-I-A^b		—	13.0 (4%)	12.2 (7%)§	—	18.5 (29%)	—
		Anti-I-A^k		—	7.7 (43%)	9.3 (30%)	—	24.3 (7%)	—
		Anti-I-A^b + α I-A^k		—		9.2 (30%)	—	—	—
			Stimulus:	K^b	K^k, I-A^k	—	—	none	H-2^k
2	bm1 Lyt-2$^+$	—		40.0	69.3	—	—	0.2	47.4
		Anti-K^bD^b		1.9 (96%)	67.9 (2%)	—	—	—	55.5 (0%)

* mAb were added to the cultures in three different dilutions. For simplicity the results obtained with only one concentration are shown, the final concentrations of the mAb being 4% for anti-I-A^b (BP107, protein A–purified ascites fluid), 0.16% for anti-I-A^k (11-5.2, unfractionated ascites fluid) and 0.2% for anti-K^bD^b (20-8-4s, protein A–purified ascites fluid) (Materials and Methods). Higher concentrations of anti-stimulator anti-I-A mAb virtually abolished the response of L3T4$^+$ cells but only marginally increased the inhibition of the response of Lyt-2$^+$ cells.

‡ Mean of triplicate cultures; for simplicity, SD have been omitted. Cells cultured in 0.5% NMS; MLR harvested on day 4.

§ Definite inhibition here was observed in other experiments.

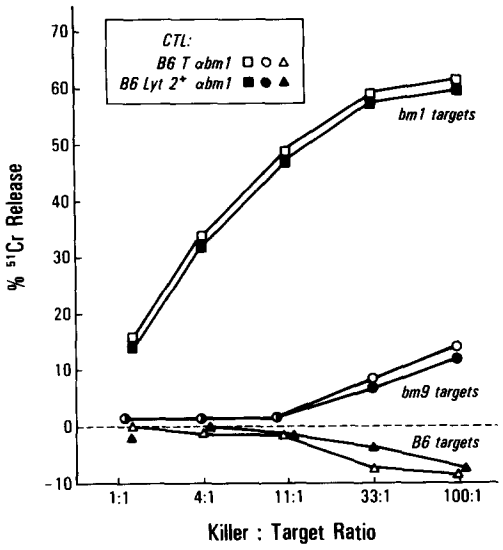

FIGURE 4. Cytolytic activity of B6 T cells or B6 Lyt-2$^+$ cells cultured for 4 d with bm1 stimulator cells pretreated with anti-Thy-1 mAb + C′. CTL activity was assayed over 3 h using ^{51}Cr-labeled Con A–stimulated blast cells as targets. Spontaneous ^{51}Cr release from target cells cultured alone was 10–20%. Each point represents the mean lysis obtained for triplicate cultures; SD were within 10–15% of the mean.

determinants on B10.A(4R) cells, despite the fact that the anti-K^bD^b mAb was presumably able to bind to the D^b determinants on these cells (as well as to the D^b determinants on the bm1 responder cells).

CTL Generated from Purified Lyt-2$^+$ Cells. In all of the experiments considered above, the function of Lyt-2$^+$ cells was assayed by MLR, i.e., by proliferation. In two experiments B6 Lyt-2$^+$ cells were tested for their capacity to generate CTL against bm1 stimulators (see Materials and Methods); unseparated T cells were used as controls. CTL activity was measured on day 4 (the time of peak responses for MLR). In both experiments, one of which is illustrated in Fig. 4, the anti-bm1 CTL activities generated from these two populations were virtually indistinguishable. Crossreactive lysis on bm9 target cells was low and there was no lysis of syngeneic targets, implying that lysis of the bm1 targets was antigen specific. Stimulator cells were pretreated with anti-Thy-1 mAb plus C′ to limit the possibility of exogenous IL-2 production.

Discussion

The main finding in this paper is that purified populations of Lyt-2$^+$ cells give high primary MLR and CTL responses to class I differences in vitro in the apparent absence of help provided by Lyt-2$^-$ cells. The evidence that Lyt-2$^-$ Th cells are not required for anti–class I MLR can be summarized as follows: First, contamination of the responding Lyt-2$^+$ cells with L3T4$^+$ cells was extremely low, indeed undetectable by FACS analysis (Table I). Second, removal of Thy-1$^+$ cells from the stimulator population failed to diminish anti–class I MLR mediated by Lyt-2$^+$ cells. Third, anti–class I MLR were not inhibited by addition of anti-L3T4 mAb to the cultures; anti-Lyt-2 mAb abolished the response.

The lack of evidence for involvement of L3T4$^+$ cells in anti–class I MLR is in accordance with recent studies of von Boehmer et al. (10) but in apparent disagreement with the findings of Rock et al. (18) and Weinberger et al. (19). These latter workers concluded that anti–class I MLR reflect Ia-restricted Th cell recognition of allo class I molecules seen in the context of self class II molecules. The main evidence for this conclusion stems from the finding of the authors (18, 19) that the failure of unseparated B6 T cells to respond to anti-Ia plus C′–treated bm1 stimulators could be partly overcome by adding syngeneic (B6) APC. Since the response was abolished by pretreating the responders with anti-Lyt-1 mAb plus C′ (anti-L3T4 mAb was not used), the authors concluded (18, 19) that the partial restoration of the response reflected antigen-processing, allo class I molecules being adsorbed by syngeneic APC and presented in association with self class II molecules. A problem with this interpretation, which the authors acknowledged, is that the responding T cells were positive for Lyt-2 as well as Lyt-1 molecules. The evidence in the present paper is difficult to reconcile with this hypothesis (18, 19). First, cell-for-cell, purified Lyt-2$^+$ cells gave higher responses to class I differences than unseparated T cells. Second, purified L3T4$^+$ cells gave no detectable response to class I differences. Third, the failure of purified Lyt-2$^+$ cells to respond to anti-Ia plus C′–treated stimulators could not be restored by addition of syngeneic APC (Table VII); only minimal (~15%) restoration of the response occurred with addition of "help" in the form of IL-2. Fourth, anti–class I MLR did not require Ia compatibility between the responders and stimulators, i.e., there was no apparent requirement for self-Ia–bearing cells.

Recent work of Singer et al. (30) provides a partial resolution to the discrepancy in the results mentioned above. This group maintains that, in the case of B6 anti-bm1 CTL, two different mechanisms govern the activation of CTL precursors. Under normal conditions, CTL generation is considered to reflect "help" provided by Ia-independent T cells; the authors quote unpublished data indicating that these cells are L3T4$^-$ (cited in 31). However, the authors provide evidence that, under artificial conditions (presentation of antigen by Ia$^+$ B cells rather than Ia$^+$ macrophage/dendritic cells), CTL generation can be controlled by Ia-restricted L3T4$^+$ cells. Although this second mechanism does not fully account for the findings of Rock et al. (18) and Weinberger et al. (19), the first mechanism proposed by Singer et al. (30) is clearly in line with the present findings. (The issue of whether the response of Lyt-2$^+$ cells depends on help from a helper-independent subset of Lyt-2$^+$ cells will be considered later.)

Although it is quite likely that Ia-restricted L3T4$^+$ cells can provide help for Lyt-2$^+$ cells under certain artificial circumstances (and perhaps also in late primary responses[2]), the point to be emphasized is that under normal conditions Lyt-2$^+$ cells respond well to class I differences in the absence of L3T4$^+$ cells. This conclusion raises three questions: (*a*) Why do primary MLR to class I differences require Ia$^+$ cells? In view of the failure to implicate L3T4$^+$ cells in

[2] The prevailing aim in the present studies was to examine the induction phase of the response of Lyt-2$^+$ cells. For this reason CTL activity was examined early in the response, i.e., on day 4. The possibility that L3T4$^+$ Th cells might amplify late (e.g., day 6) primary CTL responses has certainly not been excluded (and indeed is highly likely).

controlling responses to class I differences, it might seem surprising that pretreatment of stimulator cells with anti-Ia mAb plus C′ abolished the response of purified Lyt-2$^+$ cells; other groups have made similar findings using unseparated T cells as responders (18, 19, 30). Although various ad hoc models could be put forward to account for this paradox, perhaps the simplest explanation is that Ia$^+$ cells express a second signal required for the induction of Lyt-2$^+$ cells (32); membrane-bound IL-1 (33) is an obvious candidate for such a signal. But one still has to account for the capacity of anti-Ia mAb to block anti–class I MLR in the absence of C′. The literature on this subject is confusing, marked inhibition being noted by some workers (18, 19), but not by others (except in contrived situations) (30); unseparated T cells were used in these studies. With purified Lyt-2$^+$ cells as responders, the blocking effects of anti-Ia mAb in our hands were relatively mild and demonstrable only when MLR were of low magnitude (Table VIII). Significantly, inhibition was only seen with anti-stimulator, not anti-responder mAb. Although we do not have a cogent explanation for the blocking effects of anti-Ia mAb on anti–class I MLR, our working hypothesis is that binding of anti-Ia mAb to Ia determinants on the stimulator cells somehow down-regulates the expression of the second signal mentioned above. Although such down-regulation might not be unique to anti-Ia antibodies, it is of interest that binding of anti–class I mAb to "bystander" stimulator determinants (determinants not recognized by the responders) failed to cause inhibition (Table VIII).

(*b*) How does one account for the dogma that MLR are directed largely to class II rather than class I differences? Four points are worth making here. First, several groups (14, 18, 39) have observed appreciable responses to allelic class I differences when LN cells or purified T cells rather than spleen cells are used as responders. Second, the ratio of Lyt-2$^+$ to L3T4$^+$ cells in normal T cell populations is usually quite low (1:2). Enrichment for Lyt-2$^+$ cells, i.e., for class I–reactive cells, leads to a substantial increase in the response to allelic class I differences (Table IV). Third, it is important to emphasize that the kinetics of MLR to class I vs. class II differences are very different. In the case of L3T4$^+$ cells, the response to class II differences increased exponentially until day 6. With Lyt-2$^+$ cells, by contrast, responses to both mutant and whole H-2 differences peaked much earlier, usually at day 3–4, and then fell sharply (perhaps reflecting destruction of the stimulator cells by newly generated CTL). The peak of the response was delayed slightly by using very low doses of responders, e.g., 5×10^4, or by culturing cells in NMS, but responses were invariably very low by day 6. Measuring MLR only on day 5 or later can thus give the false impression that class I differences elicit only weak MLR. When responses are measured early in the response, e.g. at day 3–4, MLR of Lyt-2$^+$ cells to class I differences are often higher than L3T4$^+$ anti–class II MLR. A final point worth mentioning is that the conclusion that Lyt-2$^+$ cells give high MLR to class I differences rests on studies with mice of the B6 and B10 backgrounds. Interestingly, preliminary studies have shown that MLR of Lyt-2$^+$ cells to whole H-2 differences are appreciably lower with CBA/Ca and DBA/2 cells than with B6 cells. The significance of this apparent strain variation is unclear.

(*c*) Are all unprimed Lyt-2$^+$ cells helper independent? Some Lyt-2$^+$ T cell clones are known to be helper independent (HI) in that the cells respond to

specific antigen in the absence of exogenous IL-2 (8–10, 35). In some instances these clones synthesize their own IL-2 (35, 36); other clones are helper dependent (HD), i.e., require exogenous IL-2. Since T cell clones can change from being HI to HD in vitro (10), it is conceivable that all unprimed Lyt-2^+ cells are initially HI cells but then gradually become HD as the cells differentiate (10). Thus, Lyt-2^+ cells might initially produce their own help (IL-2) but then tend to become dependent on help provided by other cells, either L3T4^+ cells or HI Lyt-2^+ cells. According to this notion, in contrast to secondary responses one might expect primary responses of Lyt-2^+ cells to be relatively independent of exogenous help. The evidence that unprimed Lyt-2^+ cells can cause lethal GVHD to minor histocompatibility antigens in irradiated mice (12) and give high primary MLR and CTL responses to allo class I differences (this paper) is certainly consistent with this possibility.

It does not necessarily follow that all primary responses of Lyt-2^+ cells are HI. In this respect Singer (personal communication) has found that although generation of primary CTL responses to most mutant class I differences are HI, the response of B6 T cells to the bm6 mutant is heavily dependent on exogenous help from L3T4^+ cells. Likewise, exogenous helper cells seem to be required for priming Lyt-2^+ CTL specific for the H-Y antigen (37). These "exceptions," however, might simply reflect a very low precursor frequency of the responder cells: the latter might be HI initially but be incapable of expansion to detectable levels without help from other cells. This explanation cannot account for the data on the response to the bm6 mutant, however, since the CTL precursor frequency for B6 anti-bm6 responses is reported to be almost as high as for B6 anti-bm1 responses (38). Although we have not studied CTL responses to bm6, it is of interest that MLR of B6 Lyt-2^+ cells to the apparently identical mutant, bm9, are far lower than to the other mutants studied (Table III). In the light of these findings, it is quite possible that unprimed Lyt-2^+ cells comprise a mixture of HI and HD cells, the ratio of HI to HD cells being high for responses to bm1 (also bm4 and bm11, Table III) but low for bm9 (and perhaps antigens such as H-Y). Although unprimed HD Lyt-2^+ cells could represent a separate lineage, HD cells might arise from HI cells in vivo as the result of repeated contact with cross reactive environmental antigens.

As a final comment on "help," it should be noted that although we favor the notion that most cells participating in B6 anti-bm1 responses are HI cells, direct evidence on this point is lacking. Thus, on a priori grounds one could argue that the majority of the Lyt-2^+ cells participating in primary B6 anti-bm1 responses are HD cells, the differentiation of these cells being controlled by a minor population of IL-2-producing HI Lyt-2^+ cells. Limiting dilution analysis will be needed to assess this possibility.

One of the main aims in this paper was to assess the stringency of the role of T cell accessory molecules in controlling recognition of H-2 molecules. It has been suggested by other workers (39) that Lyt-2 and L3T4 molecules play a highly selective role in guiding T cell recognition of class I and class II molecules, respectively. The present data are in close agreement with this notion. Thus, purified Lyt-2^+ cells responded only to allo class I and not class II differences whereas L3T4^+ cells responded solely to class II differences. It should be noted

that although responses of purified Lyt-2$^+$ cells to class II differences were generally undetectable, very low responses were occasionally seen. Interestingly, these marginal responses could be inhibited either by anti-L3T4 or anti-Lyt-2 mAb (Table V). One explanation for this finding is that some Lyt-2$^+$ cells do have class II reactivity (see 40) but, unlike class I–reactive cells, these class II–restricted Lyt-2$^+$ cells are heavily dependent on help from L3T4$^+$ cells; very minor contamination of the cultures with L3T4$^+$ cells could thus allow these HD Lyt-2$^+$ cells to expand. In support of this possibility we have found (unpublished data) that bulk cultures of unseparated B6 T cells stimulated against bm12 cells contain quite a high proportion (30%) of Lyt-2$^+$ blasts. Whether these Lyt-2$^+$ blasts are specific by bm12 or are stimulated nonspecifically is unclear. In the reciprocal situation, stimulation of unseparated B6 T cells with bm1 cells generates only Lyt-2$^+$ and not L3T4$^+$ blasts (unpublished data).

The data in this paper are concerned solely with primary responses measured in vitro. In a subsequent paper we shall demonstrate that purified Lyt-2$^+$ cells respond well to allo class I molecules in vivo by a number of parameters, including proliferation, GVHD, and skin graft rejection.

Summary

In light of the widely accepted view that Ia-restricted L3T4$^+$ T helper cells play a decisive role in controlling the differentiation of Lyt-2$^+$ cells, experiments were designed to examine whether Lyt-2$^+$ cells can respond to antigen in the absence of L3T4$^+$ cells. The results showed that highly purified Lyt-2$^+$ cells gave high primary mixed lymphocyte reactions (MLR) to various class I differences, including both mutant and allelic differences; responses to class II (Ia) differences were generally undetectable with Lyt-2$^+$ cells. The intensity of MLR to class I differences was not affected by addition of anti-L3T4 monoclonal antibodies (mAb) to the cultures or by removing T cells from the stimulator populations. Negative selection experiments showed that Lyt-2$^+$ cells could respond to class I differences across Ia barriers. MLR of purified Lyt-2$^+$ cells peaked on days 3–4 and then fell sharply; background responses with syngeneic stimulators (auto-MLR) were virtually absent. Parallel experiments with purified L3T4$^+$ cells showed that this subset (*a*) responded in MLR only to class II (Ia) and not class I differences, (*b*) reached peak responses only on day 6 rather than days 3–4, and (*c*) often gave high auto-MLR. Within the first 3–4 d of culture, MLR were generally higher with Lyt-2$^+$ cells than L3T4$^+$ cells.

Although no evidence could be found that Ia-restricted L3T4$^+$ cells were required for the response of Lyt-2$^+$ cells, presentation of antigen by Ia$^+$ cells appeared to be essential. Thus, responses were ablated by pretreating stimulator cells with anti-Ia mAb plus C′. Significantly the failure of Lyt-2$^+$ cells to respond to anti-Ia plus C′–treated stimulators could not be restored by adding syngeneic spleen cells; addition of IL-2 led to only a minor (15%) restoration of the response. It is suggested that Ia$^+$ cells provide an obligatory second signal required by Lyt-2$^+$ cells.

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