

PROPERTIES OF PURIFIED T CELL SUBSETS

II. In Vivo Responses to Class I vs. Class II H-2 Differences

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Until recently, there has been little resistance to the notion that the differentiation of T killer cells, i.e. cells of the L3T4⁻, Lyt-2⁺ phenotype, is heavily dependent on help provided by other T cells (1-3). In certain situations, however, purified unprimed Lyt-2⁺ cells give high MLR and cell-mediated lympholysis (CML)¹ when exposed to H-2 differences in vitro (4, 5). By employing the bm1 and bm12 mutant mouse strains, i.e. strains differing from the C57BL/6 (B6) (H-2^b) strain only at class I (H-2K) or class II (I-A) loci, respectively, it was found that B6 Lyt-2⁺ cells responded selectively to allo-class I differences, whereas B6 L3T4⁺ cells (cells of T helper phenotype) responded only to class II differences (5). In the case of Lyt-2⁺ cells, MLR to class I (bm1) differences were not reduced by adding anti-L3T4 mAb to the cultures, or by removing T cells from the stimulator population. These findings, which are consistent with recent studies of Singer et al. (6), suggest that primary responses of Lyt-2⁺ cells can take place in the absence of Lyt-2⁻ T cells, at least in vitro. This paper presents evidence that, as in vitro, purified Lyt-2⁺ cells can mediate alloaggressive functions in vivo.

Materials and Methods

Mice. C57BL/6Kh (B6), B6.C-H-2^{bm1} (bm1), B6.C-H-2^{bm12} (bm12), B10.BR, BALB.K, BALB/c, B10.D2, CBA/Ca (CBA), and F₁ hybrid mice were obtained from the breeding colony of the Research Institute of Scripps Clinic.

Irradiation. A Gamma Cell 1,000 irradiator (500 rad/min) and a Gamma Cell 40 irradiator (90 rad/min) (both ¹³⁷Cs sources from Atomic Energy of Canada, Ottawa, Canada) were used to irradiate cells and mice, respectively.

Media. As described previously (5), RPMI 1640 or HBSS supplemented with either 5% FCS or γ -globulin-depleted horse serum were used. For MLR, cells were cultured in vitro in RPMI 1640 with 10% FCS plus standard additives (5).

Monoclonal Antibodies. Anti-L3T4 (GK1.5, ascites fluid), anti-Lyt-2 (3.168.8, ascites fluid), anti-Thy-1.2 (J1j, ascites fluid), and anti-B (J11d, culture supernatant) mAb and guinea pig serum as a source of C' were used as described previously (5).

Purification of T Cell Subsets. As described in detail elsewhere (5), purified T cell subsets were obtained by subjecting pooled lymph node (LN) cells to mAb plus C', followed by positive panning on mAb-coated dishes. To make Lyt-2⁺ cells, LN cells were

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¹Abbreviations used in this paper: CML, cell-mediated lympholysis; LN, lymph node; TDL, thoracic duct lymphocyte.

treated with a mixture of anti-B (J11d) and anti-L3T4 mAb plus C', washed, and then allowed to adhere to anti-Lyt-2-coated dishes. After gently washing nonadherent cells from the dishes, the adherent cells were eluted by vigorous pipetting after incubation at 4°C for 1 h. The eluted cells were >99% Lyt-2⁺ cells by FACS analysis, and contained no detectable L3T4⁺ cells. An analogous procedure was used to prepare >99% pure L3T4⁺ cells, i.e. pretreatment of LN cells with J11d and anti-Lyt-2 mAb plus C', followed by positive panning on anti-L3T4-coated dishes.

MLR. Doses of $1-2 \times 10^5$ 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 μ l, and then pulsed with 1 μ Ci [³H]TdR 18 h before harvest (5).

T Cell Proliferation In Vivo. Using a technique described previously (7), T cells were transferred to allogeneic or syngeneic mice exposed to heavy irradiation (950-1,000 rad) 4-6 h before. At various times thereafter, the mice were injected intravenously with 25 μ Ci [³H]TdR and killed 45 min later. The spleens were dissolved in Soluene 100 (Packard Instrument Co., Downers Grove, IL) overnight, and counted in a β -counter after addition of scintillation fluid. Background counts in spleens of irradiated mice given [³H]TdR but no lymphoid cells (subtracted from the data shown) were $25-30 \times 10^3$ cpm.

Thoracic Duct Lymphocytes (TDL). Cells emerging through a cannula placed in the thoracic duct were collected as described elsewhere (8).

Measurement of Splenomegaly in Newborn Mice. A modification of the technique of Simonsen (9) was used. T cells were transferred intraperitoneally in varying doses into 1-2-d-old neonatal mice. The recipients were killed 9-10 d later to measure the weight of the spleen vs. body weight. Splenic indices were calculated with respect to spleen/body weight ratios in control mice, i.e. mice given 10^6 unseparated syngeneic T cells (J11d plus C'-treated LN). The formula for calculating splenic indices was: (spleen-to-body weight ratio for each mouse of experimental group)/(mean spleen-to-body weight ratio of mice given syngeneic T cells).

Assay for Lethal GVHD. Adult mice aged 10-15 wk were exposed to heavy irradiation (950 rad) 4-6 h before transfer of T cells and anti-Thy-1 plus C'-treated marrow cells given intravenously. Mice were inspected three times per week until death. No antibiotics were given to the mice.

Skin Grafting. Ear skin grafts were applied to the flank region by the method of Billingham (10).

Typing of Cells. Cells were typed for cell surface expression of Thy-1, L3T4, and Lyt-2 markers by FACS analysis or cytotoxicity as described previously (5).

Results

In all of the experiments presented below, >99% purified L3T4⁺ and Lyt-2⁺ cells were prepared by a combination of killing and panning (Materials and Methods).

MLR In Vitro. In prior studies (5), the evidence on MLR mediated by L3T4⁺ and Lyt-2⁺ cells rested largely on experiments with B6 and B10.A mice. Before studying the function of T cell subsets in vivo, it was therefore considered important to examine whether the responsiveness of Lyt-2⁺ cells in MLR extended to other responder strains. Table I compares MLR by T cell subsets taken from five different strains; stimulators were pretreated with anti-Thy-1 mAb plus C'.

L3T4⁺ cells from all strains tested gave high MLR to full H-2 differences, with peak responses usually occurring on day 6. Strain variation in peak responses was comparatively minor. The responsiveness of B6 L3T4⁺ cells to class II (bm12) but not class I (bm1) differences (5 and Table I) suggests that anti-H2 responses by L3T4⁺ cells are directed selectively to class II differences.

As found previously with B6 cells (5), MLR by the five populations of Lyt-2⁺

TABLE I
Comparative MLR of L3T4⁺ and Lyt-2⁺ Cells from Various Strains of Mice

Exp.	Responders*	Number of responders	Stimulators (anti-Thy-1 + C'-treated) [‡]	Stimulus	[³ H]TdR incorporation (cpm × 10 ³) [§]		
					Day 4	Day 5	Day 6
1	B6 L3T4 ⁺	10 ⁵	B6	Self	1.0	1.6	2.2
			bm1	K ^{bm1}	3.3	3.7	6.1
			bm12	I-A ^{bm12}	25.4	32.6	98.2
			(B6 × CBA)F ₁	H-2 ^k	41.6	56.0	145.0
	B6 Lyt-2 ⁺	B6	Self	0.2	0.2	0.1	
		bm1	K ^{bm1}	99.7	92.8	2.4	
		bm12	I-A ^{bm12}	0.8	0.8	2.5	
		(B6 × CBA)F ₁	H-2 ^k	84.6	53.2	1.7	
2	CBA L3T4 ⁺	2 × 10 ⁵	CBA	Self	2.5	2.0	4.7
			(B6 × CBA)F ₁	H-2 ^b	55.2	159.0	327.0
	CBA Lyt-2 ⁺	CBA	Self	0.1	0.2	0.1	
		(B6 × CBA)F ₁	H-2 ^b	11.2	17.6	10.1	
	B10.BR L3T4 ⁺	2 × 10 ⁵	CBA	Self	1.5	2.1	3.2
			(B6 × CBA)F ₁	H-2 ^b	85.2	196.9	100.2
	B10.BR Lyt-2 ⁺	CBA	Self	0.7	0.3	0.6	
		(B6 × CBA)F ₁	H-2 ^b	42.3	29.0	3.9	
3	BALB/c L3T4 ⁺	2 × 10 ⁵	BALB/c	Self	5.5	11.1	21.6
			(BALB/c × BALB.K)F ₁	H-2 ^k	49.7	120.3	170.6
	BALB/c Lyt-2 ⁺	BALB/c	Self	0.4	0.2	0.2	
		(BALB/c × BALB.K)F ₁	H-2 ^k	40.9	16.4	1.0	
	B10.D2 L3T4 ⁺	2 × 10 ⁵	BALB/c	Self	6.0	7.2	12.4
			(BALB/c × BALB.K)F ₁	H-2 ^k	59.0	163.1	37.6
	B10.D2 Lyt-2 ⁺	BALB/c	Self	0.4	0.4	0.2	
		(BALB/c × BALB.K)F ₁	H-2 ^k	75.4	30.7	3.7	

* H-2 haplotype of responders: B6, H-2^b; CBA and B10.BR, H-2^k; BALB/c and B10.D2, H-2^d.

[‡] 5 × 10⁵ viable cells.

[§] Mean of triplicate cultures. SD, omitted for simplicity, were generally within 10–20% of the mean.

cells to full H-2 differences peaked quite early, usually on day 4, and then fell sharply to near-background levels by day 6; background counts with syngeneic stimulators were uniformly negligible. Since B6 Lyt-2⁺ cells are unresponsive to mutant class II differences (5 and Table I), it seems likely that anti-H-2 MLR by Lyt-2⁺ cells are directed selectively to class I differences. In contrast to L3T4⁺ cells, MLR of Lyt-2⁺ cells showed considerable strain variation, with very high responses produced by B6 cells and only low responses by CBA cells. At day 4, MLR with B6 cells were appreciably higher with Lyt-2⁺ than L3T4⁺ cells; the reverse applied with CBA cells, the response of L3T4⁺ cells far exceeding the Lyt-2⁺ response. In the case of the other three strains tested, B10.BR, BALB/c, and B10.D2, MLR by L3T4⁺ and Lyt-2⁺ cells on day 4 were roughly comparable.

Because of the availability of the bm1 and bm12 mutant strains, B6 T cell subsets were used for most of the in vivo experiments described below. Emphasis was also placed on studying CBA Lyt-2⁺ cells in vivo, since these cells gave

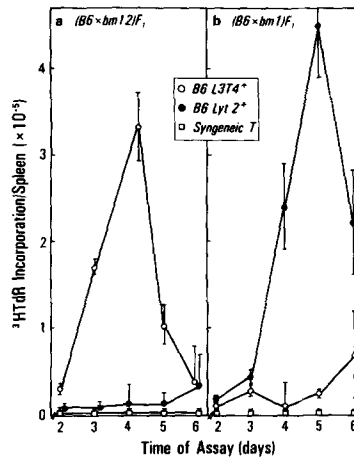


FIGURE 1. Incorporation of [^3H]TdR in spleens of irradiated (B6 \times bm12) F_1 (a) and (B6 \times bm1) F_1 (b) mice injected with 0.5×10^6 B6 L3T4 $^+$, B6 Lyt-2 $^+$, or unseparated syngeneic T cells, given intravenously. Groups of mice were injected with [^3H]TdR and killed at daily intervals, 45 min after [^3H]TdR injection (see text for details). The data show the mean levels of radioactivity (\pm SD) recovered from the spleens of the recipients (3 mice/group) after subtraction of background counts (Materials and Methods).

relatively poor responses in vitro. The low MLR of CBA Lyt-2 $^+$ cells to full H-2 differences in vitro is not blocked by anti-L3T4 mAb (our unpublished results).

Proliferative Responses In Vivo. T cell proliferation in vivo can be quantitated by transferring T cells into heavily irradiated mice and then pulsing the mice with [^3H]TdR shortly before removing the spleen (Materials and Methods). At 4 d postinjection, levels of radioactivity in the spleen are directly proportional to the number of T cells injected, within the range of 10^5 – 10^6 cells (11); provided that the T cells are prepared from tissues, such as LN, that lack stem cells, the cells proliferating in the spleen of the recipients are almost entirely donor-derived T cells.

The kinetics of the response of B6 T cell subsets in irradiated (950 rad) (B6 \times bm1) F_1 and (B6 \times bm12) F_1 mice is shown in Fig. 1; background counts observed in irradiated mice given no cells have been subtracted from the data (Materials and Methods). B6 L3T4 $^+$ cells responded strongly in bm12 F_1 mice, but gave little if any response in bm1 F_1 mice, relative to control bm1 F_1 T cells. The reverse applied to B6 Lyt-2 $^+$ cells. These cells gave strong responses in bm1 F_1 mice but virtually no response in bm12 F_1 mice. Peak responses occurred slightly earlier for B6 L3T4 $^+$ cells (day 4) than for B6 Lyt-2 $^+$ cells (day 5).

When B6 T cell subsets were transferred to H-2 semiallogeneic irradiated (B6 \times CBA) F_1 mice, only quite low responses were obtained, both for L3T4 $^+$ and Lyt-2 $^+$ cells (Table II). Hh resistance (12) probably accounted for these low responses, since split-dose irradiation of the recipients, a procedure known to overcome Hh resistance, led to appreciably higher responses (Table II). In the case of H-2 k responder cells [which are not subject to Hh resistance in (b \times k) F_1 mice], CBA and B10.BR L3T4 $^+$ cells both gave high responses in (B6 \times CBA) F_1 mice. Intermediate responses were observed with B10.BR Lyt-2 $^+$ cells. CBA Lyt-

TABLE II
Proliferative Response of Parental Strain L3T4⁺ and Lyt-2⁺ Cells in Irradiated
(B6 × CBA)F₁ Mice

Cells injected	Irradiated recipients	Radiation dose (rad)	³ H]TdR incorporation (cpm × 10 ³)/spleen (SD)	
			Day 4	Day 5
(B6 × CBA)F ₁ T	(B6 × CBA)F ₁	950	8.2 (0.7)	0.5 (0.6)
(B6 × CBA)F ₁ T	(B6 × CBA)F ₁	550 + 950	3.2 (4.6)	3.7 (1.2)
B6 L3T4 ⁺	B6	950	10.2 (3.1)	—
B6 L3T4 ⁺	(B6 × CBA)F ₁	950	108.8 (16.2)	185.7 (30.4)
B6 L3T4 ⁺	(B6 × CBA)F ₁	550 + 950	240.7 (35.4)	266.3 (40.9)
B6 Lyt-2 ⁺	B6	950	0.2 (0.5)	—
B6 Lyt-2 ⁺	(B6 × CBA)F ₁	950	59.4 (1.9)	100.2 (20.3)
B6 Lyt-2 ⁺	(B6 × CBA)F ₁	550 + 950	119.5 (22.2)	186.4 (32.1)
CBA L3T4 ⁺	CBA	950	13.8 (3.4)	—
CBA L3T4 ⁺	(B6 × CBA)F ₁	950	527.9 (25.7)	256.9 (33.2)
CBA Lyt-2 ⁺	CBA	950	3.5 (2.7)	—
CBA Lyt-2 ⁺	(B6 × CBA)F ₁	950	29.9 (7.3)	41.0 (10.1)
B10.BR L3T4 ⁺	CBA	950	22.3 (5.9)	—
B10.BR L3T4 ⁺	(B6 × CBA)F ₁	950	735.4 (117.5)	633.6 (92.4)
B10.BR Lyt-2 ⁺	CBA	950	18.2 (12.3)	—
B10.BR Lyt-2 ⁺	(B6 × CBA)F ₁	950	86.0 (15.6)	301.7 (12.2)

0.5 × 10⁶ T cells were transferred intravenously to mice irradiated 4 h before; mice given split-dose irradiation received the first dose of radiation 2 wk before cell transfer and the second dose 4 h before cell transfer. The recipients were killed 4 or 5 d posttransfer, 45 min after receiving 25 μCi [³H]TdR intravenously. Radioactivity in the spleens of the recipients was measured as described in Materials and Methods. The data are shown as mean radioactivity counts (SD) from three mice per group. Background counts have been subtracted (Materials and Methods).

2⁺ cells gave only very low responses. As for Fig. 1, peak responses were observed on day 5 for Lyt-2⁺ cells and on day 4 for L3T4⁺ cells.

TDL Outputs. T cell proliferation in the lymphoid tissues of irradiated H-2-incompatible mice is followed by entry of large numbers of blast T cells into the central lymph (13). With transfer of small numbers of T cells (<5 × 10⁶), the number of blasts entering the lymph is proportional to the number of T cells injected. Counting T blasts in thoracic duct lymph thus provides direct information on the extent of donor T cell proliferation in the recipients.

The data in Table III show TDL outputs in irradiated (950 rad) bm1 F₁ and bm12 F₁ mice given 3 × 10⁶ unseparated B6 T cells or B6 T cell subsets. The recipients were cannulated at 4 d posttransfer, and TDL were collected over the first 24 h of drainage; cells collected over the first 14–18 h were typed for expression of Thy-1, L3T4, and Lyt-2 markers, usually by FACS analysis.

Whereas normal mice produce 5–15 × 10⁷ T cells over a 24-h drainage period (8), TDL outputs in irradiated mice are extremely low, i.e. <10⁶ cells/24 h (groups 1 and 6). Most of the residual cells in the lymph are resting radioresistant Thy-1⁺, L3T4⁺ cells, Lyt-2⁺ cells being slightly more radiosensitive than L3T4⁺ cells (14); B cells, which are highly radiosensitive, are quite rare. With transfer

TABLE III
TDL Outputs in Irradiated Mice Given L3T4⁺ vs. Lyt-2⁺ Cells

Group	Donor cells (3×10^6)	Irradiated (950 rad) hosts	Mice/group (<i>n</i>)	TDL ($\times 10^6$) collected over 24 h (SD)	Percent cells positive for:*		
					Thy-1	L3T4	Lyt-2
1	—	(B6 \times bm1)F ₁	5	0.7 (0.3)	92.1	64.1	13.9
2	(B6 \times bm1)F ₁ T	(B6 \times bm1)F ₁	5	2.3 (0.3)	99	48	48
3	B6 L3T4 ⁺	(B6 \times bm1)F ₁	5	1.4 (0.2)	97.8	91.9	3.6
4	B6 Lyt-2 ⁺	(B6 \times bm1)F ₁	4	22.6 (4.6)	99.1	1.3	98.6
5	B6 T	(B6 \times bm1)F ₁	3	12.4 (2.9)	98.6	5.9	92.4
6	—	(B6 \times bm12)F ₁	5	0.7 (0.2)	93.5	62.1	16.1
7	(B6 \times bm12)F ₁ T	(B6 \times bm12)F ₁	4	1.5 (0.4)	98	53	46
8	B6 L3T4 ⁺	(B6 \times bm12)F ₁	3	29.7 (3.4)	99.7	99.5	0.9
9	B6 Lyt-2 ⁺	(B6 \times bm12)F ₁	5	1.7 (1.2)	96.6	20.5	75.3
10	B6 T	(B6 \times bm12)F ₁	4	21.6 (3.1)	98.5	88.5	14.1
11	(B6 \times CBA)F ₁ T	(B6 \times CBA)F ₁	3	1.6 (0.3)	100	61	36
12	CBA L3T4 ⁺	(B6 \times CBA)F ₁	3	16.9 (2.8)	100	99	1
13	CBA Lyt-2 ⁺	(B6 \times CBA)F ₁	4	6.1 (0.6)	100	5	93
14	B10.BR Lyt-2 ⁺	(B6 \times CBA)F ₁	5	12.7 (2.6)	100	2	94

Mice were injected intravenously with 3×10^6 viable cells, either purified T cell subsets or whole T cells (J11d-treated LN); cells were injected within 4–6 h of exposing the recipients to irradiation. Mice were cannulated 4 d posttransfer, and TDL were collected over the following 24 h. In most cases, FACS analysis was used to quantitate expression of cell surface markers, using aliquots of TDL collected over 14–16 h; TDL in groups 2, 7, and 11–14 were typed by cytotoxicity. When typing cells for expression of L3T4 molecules by cytotoxicity in groups 11–14, RL172 mAb was used (15); unlike GK1.5 mAb, RL172 mAb is highly efficient at lysing L3T4⁺ blast cells. Cytotoxic indices with antihist (anti-K^bD^b) 20-8-4s mAb (16) were 99% for group 11 cells, 1% for group 12 cells, 5% for group 13 cells, and 2% for group 14 cells.

* Cells for typing were pooled in groups 1–3, 6, 7, 9, and 11. For the other groups, TDL from 2–3 individual mice were tested; for these groups the mean of the results is shown.

of syngeneic T cells into irradiated mice, TDL outputs increased two- to threefold, the lymph-borne cells consisting of a 1:1 ratio of L3T4⁺/Lyt-2⁺ cells (groups 2 and 7); very few of these cells (<5%) were blast cells. In the case of irradiated bm1 F₁ recipients of B6 Lyt-2⁺ cells, very large numbers of T cells ($>2 \times 10^7$ cells/mouse) entered the lymph (group 4); these cells were nearly all blast cells, and 99% were Lyt-2⁺. In marked contrast, only very few T cells (mostly small lymphocytes) were collected from bm1 F₁ mice given B6 L3T4⁺ cells (group 3). Diametrically opposite findings were observed in bm12 F₁ mice. Here, injection of B6 L3T4⁺ cells generated very large numbers of lymph-borne T blasts (>99% L3T4⁺) (group 8), whereas injection of B6 Lyt-2⁺ cells generated very few cells (group 9), most of which were small lymphocytes. Contamination with radioresistant L3T4⁺ host cells is presumed to account for the 20% of L3T4⁺ cells in the lymph of bm12 F₁ given B6 Lyt-2⁺ cells. With transfer of unseparated B6 T cells (containing an ~1:1 ratio of L3T4⁺/Lyt-2⁺ cells) to irradiated bm1 F₁ and bm12 F₁ mice (groups 5 and 10), TDL outputs were high in both situations, and most of the cells were blasts. In bm1 F₁ recipients, the vast majority (92%) of the lymph-borne cells were Lyt-2⁺, only 6% of the cells being L3T4⁺. The reverse applied in bm12 F₁ recipients, 89% of the cells being L3T4⁺ and 14% Lyt-2⁺.

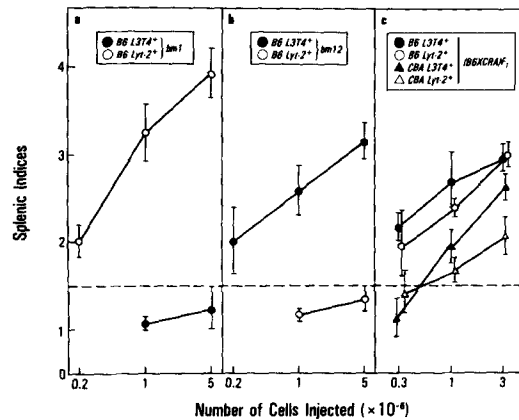


FIGURE 2. Splenomegaly induced in newborn bm1 (a), bm12 (b), and (B6 × CBA) F_1 (c) mice injected intraperitoneally with varying numbers of B6 (a–c) or CBA (c) L3T4 $^+$ or Lyt-2 $^+$ cells. The mice were killed at 9 d posttransfer to measure splenic indices (Materials and Methods); indices >1.5 (dotted line) are considered to be significant. Each point represents the mean (\pm SD) of the splenic indices calculated for 3–5 mice/group.

Table III also shows TDL outputs in irradiated (B6 × CBA) F_1 recipients of CBA L3T4 $^+$ and Lyt-2 $^+$ cells and B10.BR Lyt-2 $^+$ cells. With transfer of CBA L3T4 $^+$ cells, large numbers of cells (>90% blasts) entered the lymph (group 12). TDL outputs were lower with transfer of Lyt-2 $^+$ cells, cell yields being appreciably higher for B10.BR cells than CBA cells; in both situations, the vast majority of the lymph-borne cells (>90%) were blasts. Only very low cell yields were found in recipients of syngeneic T cells, very few of these cells (<5%) being blasts. Cytotoxicity testing with anti-host mAb confirmed that the vast majority of the lymph-borne cells in groups 12–14 were of donor origin (Table III footnote).

Splenomegaly in Neonatal Mice. The capacity of B6 L3T4 $^+$ and B6 Lyt-2 $^+$ cells to elicit splenomegaly in newborn (1–2-d-old) bm1 and bm12 mice is shown in Fig. 2, a and b; cells were injected intraperitoneally, and spleen and body weights were measured at 9 d posttransfer to calculate splenic indices. It can be seen that B6 Lyt-2 $^+$ cells produced marked splenomegaly in bm1 mice, but no splenic enlargement in bm12 mice (Fig. 2a). Conversely, B6 L3T4 $^+$ cells caused splenomegaly only in bm12 and not bm1 mice (Fig. 2b). In addition to splenomegaly, B6 L3T4 $^+$ → bm12 mice and B6 Lyt-2 $^+$ → bm1 mice both displayed macroscopic liver lesions characteristic of GVHD (data not shown). The incidence of these lesions was comparable in the two groups, i.e. 80% with the highest dose of cells injected (5×10^6).

Splenomegaly in neonatal (B6 × CBA) F_1 mice (which do not display Hh resistance) is shown in Fig. 2c. In the case of B6 T cell subsets, L3T4 $^+$ and Lyt-2 $^+$ cells both caused marked splenomegaly, the potency of these two groups of cells being roughly comparable. CBA T cell subsets also both caused significant splenomegaly, although splenic indices were appreciably higher with L3T4 $^+$ cells than with Lyt-2 $^+$ cells.

Lethal GVHD. The capacity of B6 T cell subsets to cause lethal GVHD in irradiated (950 rad) homozygous bm12 and bm1 mice is shown in Fig. 3, a and b; similar results were observed in F_1 recipients (data not shown). T cell subsets

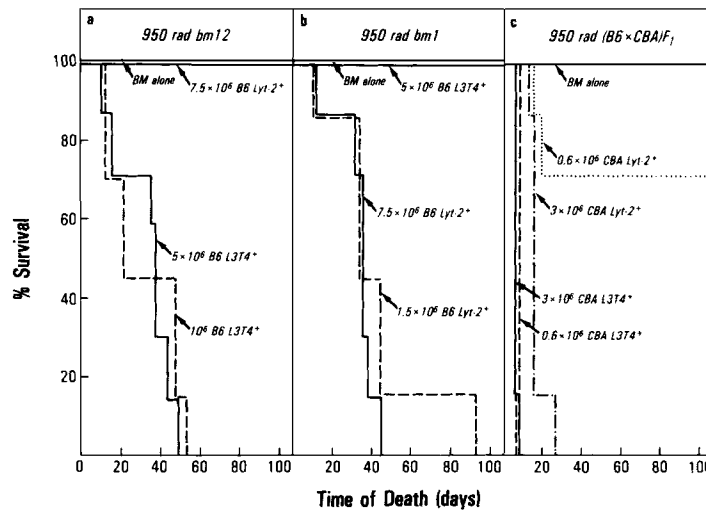


FIGURE 3. Mortality in irradiated (950 rad) bm12 (a), bm1 (b), and (B6 × CBA) F_1 (c) mice injected intravenously with varying numbers of B6 (a and b) or CBA (c) L3T4⁺ or Lyt-2⁺ cells plus 4×10^6 anti-Thy-1 plus C'–treated syngeneic marrow cells; 7–8 mice/group.

were injected intravenously together with T cell–depleted marrow cells, the marrow cells being syngeneic with the T cells; no deaths occurred in mice reconstituted with marrow cells alone. Both T cell subsets caused 100% incidence of lethal GHVD, B6 L3T4⁺ cells killing only bm12 and not bm1 mice, and B6 Lyt-2⁺ cells killing bm1 but not bm12 mice. Lethal GHVD also occurred with transfer of CBA T cell subsets to irradiated (B6 × CBA) F_1 mice (Fig. 3c). L3T4⁺ cells were highly potent, low doses of cells (0.6×10^6) causing 100% mortality by 10 d posttransfer; in other experiments, 100% mortality was observed with doses as low as 0.1×10^6 cells (data not shown). Lyt-2⁺ cells were appreciably less potent, and only the highest dose tested (3×10^6) caused severe mortality.

Rejection of Skin Allografts. To examine the capacity of B6 T cell subsets to cause skin allograft rejection, adult thymectomized B6 mice were exposed to heavy irradiation (1,000–1,100 rad) and reconstituted with anti-Thy-1 plus C'–treated syngeneic marrow cells (4×10^6) given intravenously. Thereafter the mice were injected intravenously with $8\text{--}10 \times 10^6$ L3T4⁺ or Lyt-2⁺ cells and then grafted 1–2 d later with ear skin taken from bm1 and bm12 mice; the two grafts were placed contiguously on the flank. To limit participation of radioreistant host cells in graft rejection, the mice received a large dose of anti-Thy-1 mAb 2 wk before T cell transfer (Table IV, footnote). Two experiments were performed; one (Exp. 2) was arbitrarily terminated at 31 d (the mice were then used for another experiment) and the other (Exp. 1) at 50 d; technical failures complicated graft rejection in the second experiment with L3T4⁺ cells and only the data for Lyt-2⁺ cells are shown for this experiment.

As seen in Table IV, both T cells subsets caused graft rejection. With transfer of L3T4⁺ cells, all five of the bm12 grafts were rejected rapidly with a mean survival time of 17 d; only one of five of the bm1 grafts was rejected within the observation time, and the rejection of this graft occurred quite late (38 d). With injection of Lyt-2⁺ cells, only bm1 and not bm12 grafts were rejected; eight of

TABLE IV

Rejection of bm1 and bm12 Skin Allografts by Thymectomized, Irradiated Marrow-protected B6 Mice Injected with B6 L3T4⁺ or B6 Lyt-2⁺ Cells

Exp.	Cells injected	Number of cells injected	Skin graft	Graft rejection		
				Grafts rejected/total	Day of rejection	Mean survival time (d)
1	L3T4 ⁺	8 × 10 ⁶	bm1	1/5	38	>50
			bm12	5/5	11, 14, 16, 18, 25	17
1	Lyt-2 ⁺	8 × 10 ⁶	bm1	3/3	16, 16, 18	17
			bm12	0/3	—	>50
2	Lyt-2 ⁺	10 ⁷	bm1	5/6	11, 14, 19, 23, 31	20 [‡]
			bm12	0/4*	—	>31

See text for details. In Exp. 1, mice were thymectomized 8 wk before irradiation (1,000 rad) and given T cells and skin grafts 8 wk later. In Exp. 2, mice were thymectomized 1 wk before irradiation (1,100 rad) and given T cells and skin grafts 6 wk later. A dose of 200 μl of ascites fluid (titer > 3 × 10⁶) of the anti-Thy-1 hybridoma, T24, was given to each of the mice 2 wk before T cell transfer; this mAb is highly effective at removing T cells in vivo (17).

* The other two grafts in this group were technical failures, i.e. the grafts failed to take initially.

‡ Excluding the one surviving graft (which survived >31 d).

nine of the bm1 grafts were rejected, with a mean survival time of 17 d for Exp. 1, and 20 d for Exp. 2.

Discussion

Current information on the function of purified T cell subsets in vivo has come largely from studies in rats. Other workers (18, 19) investigated alloaggressive phenomena mediated by purified W3/25⁺ and OX8⁺ cells, the homologs of L3T4⁺ and Lyt-2⁺ cells. In the case of popliteal LN enlargement, only W3/25⁺ cells caused significant responses. Both subsets, however, caused lethal GHVD in irradiated rats, although W3/25⁺ cells were more potent than OX8⁺ cells.

Since antibodies specific for mouse L3T4 molecules have only recently become available, most of the information on the function of mouse T cell subsets in vivo is indirect and is based largely on comparing the response of unseparated T cells reacting to class II vs. class I differences. There is general agreement that class II differences elicit strong alloreactivity in vivo by all parameters examined, including T cell proliferation and lethal GHVD in irradiated mice, splenomegaly induction in newborn mice, and skin graft rejection (20–26). Although class I differences generally cause rapid rejection of allografts, other forms of alloaggression to class I differences are quite variable, the intensity of these responses depending on the assay used and the particular class I differences involved. Allelic class I differences are reported to elicit minimal T cell proliferation in vivo (24), and cause only very mild GHVD in sublethally irradiated (400 rad) mice (23), although severe mortality can occur with heavily irradiated recipients (26); induction of splenomegaly in neonates varies from very low to moderate, depending on the allele concerned (25). In contrast to the generally weak alloreactivity to allelic class I differences, mutant class I differences elicit quite

strong responses as measured by induction of splenomegaly and lethal GVHD (22, 26). Why antimutant responses are so strong is unclear (22).

Although the strain combinations tested in this paper were quite limited, the results indicate that highly purified populations of L3T4⁺ and Lyt-2⁺ cells are each capable of responding to H-2 differences in vivo. The studies with bm1 and bm12 mutant strains suggest that, as in vitro (5), B6 Lyt-2⁺ cells and L3T4⁺ cells respond selectively to class I and class II differences, respectively. This selectivity applied to T cell DNA synthesis and blast cell generation in irradiated mice, induction of splenomegaly in newborn mice, lethal GVHD in irradiated mice, and skin allograft rejection. These data provide strong support for the view (27) that recognition of class I and class II molecules correlates closely with T cell expression of Lyt-2 and L3T4 molecules, respectively, at least for allorecognition. In the case of lethal GVHD, we reported previously (26) that semipurified B6 Lyt-2⁺ cells elicited severe mortality in irradiated bm1 mice but also caused some mortality in bm12 mice. The fact that the highly purified Lyt-2⁺ cells used in the present experiments failed to kill bm12 mice suggests that our previous results reflected minor contamination of the Lyt-2⁺ population with L3T4⁺ cells. With regard to skin graft rejection, the selective rejection of bm1 grafts by B6 Lyt-2⁺ cells and bm12 grafts by B6 L3T4⁺ cells suggests that the issue of whether graft rejection reflects Lyt-2⁺-mediated CTL activity or L3T4⁺-controlled delayed-type hypersensitivity (reviewed in 28) depends largely on the type of alloantigen difference (class I vs. class II) involved. The secondary issue of whether Lyt-2⁺-mediated graft rejection and other functions of Lyt-2⁺ cells are helper-independent is discussed below.

Although each of the three populations of Lyt-2⁺ cells tested gave significant responses in vivo, responses were much higher with B6 cells than CBA cells; B10.BR cells gave intermediate responses. By all parameters tested, the response of B6 Lyt-2⁺ cells to bm1 was as strong as the anti-class II response of B6 L3T4⁺ cells reacting to bm12; B6 Lyt-2⁺ cells also responded well to a full H-2 difference. The intensity of the in vivo responses of B6 Lyt-2⁺ cells correlated closely with the very high MLR displayed by these cells in vitro. Cell-for-cell, purified B6 Lyt-2⁺ cells were more potent than unseparated T cells, as manifested by splenomegaly induction (data not shown) and blast cell generation (Table III). The high responses given by B6 Lyt-2⁺ cells contrasted with the rather low responses given by CBA Lyt-2⁺ cells (reacting against a whole H-2 [K, D, and L] difference). As in vitro, CBA Lyt-2⁺ cells gave poor proliferative responses in vivo. These cells nevertheless evoked appreciable GVH reactions, and doses of 3×10^6 cells caused 100% mortality in heavily irradiated (B6 \times CBA)F₁ mice.

The reason for the marked strain variability in the response of Lyt-2⁺ cells, both in vitro and in vivo, is unclear. In rats, one group reports (29) that purified OX8⁺ cells give very high MLR in vitro, whereas another group (30), using a different strain combination, asserts that OX8⁺ cells give no response without added help. Although it is difficult to account for this striking difference in rats, we have recently found that Lyt-2⁺ cells derived from a series of H-2^b strains vary considerably in their capacity to give MLR to bm1 (unpublished data). For example, BALB.B cells give much lower responses than B6 cells. This finding

suggests that background genes somehow influence the responsiveness of Lyt-2⁺ cells. The identity of these genes is quite unknown.

In the face of the considerable strain variation in the reactivity of purified Lyt-2⁺ cells, it is difficult to reach firm conclusions on the important issue of whether the response of Lyt-2⁺ cells to antigen *in vivo* is autonomous or requires exogenous help from L3T4⁺ cells. For *in vitro* responses, we could find no evidence that contamination with L3T4⁺ cells accounted for the B6 Lyt-2⁺ anti-bm1 response, either for MLR or for early (day 4) generation of CML (5). Although the method used for preparing Lyt-2⁺ cells virtually eliminates L3T4⁺ cells from the responder population, the possibility that *in vivo* responses of Lyt-2⁺ cells reflect help from host-derived L3T4⁺ cells is less clear. It was mentioned earlier (see Results) that ~1% of the T cell pool is resistant to heavy irradiation (e.g. 950 rad), and that most of these cells are L3T4⁺ cells. Although these few radioresistant cells are obvious candidates for providing help *in vivo*, these cells are incapable of responding in MLR (at least within 5 d of irradiating the recipients), probably because the cells have sustained radiation-induced chromosomal damage (31). The possibility that these radioresistant cells might produce a minimal amount of help required by Lyt-2⁺ cells, however, cannot be discounted. Nevertheless, in view of the conspicuous lack of evidence that L3T4⁺ cells are required for Lyt-2⁺ responses *in vitro*, it seems highly likely that Lyt-2⁺ cells behave similarly *in vivo*, especially for short-term assays such as T cell proliferation in irradiated mice.

Whether exogenous help is needed for more protracted *in vivo* responses of Lyt-2⁺ cells is less clear. In the case of B6 Lyt-2⁺-mediated skin graft rejection, it seems unlikely that host-derived L3T4⁺ cells played a significant role because the (thymectomized) recipients were pretreated with heavy irradiation (1,100 rad in one experiment), and received a large dose of opsonizing anti-Thy-1 mAb before cell transfer; moreover, the recipients of Lyt-2⁺ cells did not reject bm12 grafts. It seems quite likely therefore that rejection of bm1 grafts by B6 Lyt-2⁺ cells does not depend on exogenous help. Bearing in mind that the B6 anti-bm1 response is so intense, however, it is quite possible that skin graft rejection directed to other (weaker) class I differences will prove to be heavily helper-dependent. Further studies on graft rejection by Lyt-2⁺ cells are clearly needed.

The question of whether T-T interaction is required for GVH reactions deserves careful consideration. In the case of splenomegaly induction in neonates, bystander help from newly-formed host T cells is difficult to exclude. We hope to address this question in the future by thymectomizing the recipients and/or giving the mice repeated doses of anti-L3T4 mAb. As for skin allograft rejection (see above), it might seem unlikely that host-derived help could contribute significantly to lethal GVHD mediated by Lyt-2⁺ cells in heavily irradiated mice. Surprisingly, however, very recent studies (unpublished) have suggested that exogenous help is indeed important for Lyt-2⁺-mediated GVHD. Although quite low doses of B6 Lyt-2⁺ cells cause 100% mortality in irradiated bm1 mice, most of the deaths occur quite late, i.e. after day 30, even when large numbers of cells are injected. Supplementing the Lyt-2⁺ cells with small doses of L3T4⁺ cells, however, leads to acute GVHD, with most deaths occurring before day 21 (our unpublished data). This finding suggests that the late mortality caused by

Lyt-2⁺ cells transferred alone might depend on bystander help produced by newly-generated, donor-derived L3T4⁺ cells emerging from the host thymus. To test this idea, we are currently examining whether thymectomy of the irradiated recipients prevents Lyt-2⁺ cells from causing mortality. If this were the case, the possibility would arise that although Lyt-2⁺ cells are able to function autonomously during the first 1–2 wk posttransfer, these cells eventually differentiate to a helper-dependent state; the cells revert to a resting phase, but then become reactivated when bystander help becomes available. In vitro studies of von Boehmer et al. (4) provide a precedent for this notion.

Although Lyt-2⁺ and L3T4⁺ cells both responded in each of the in vivo assays tested, the effector functions of these cells are known to be quite different, at least in vitro. Hence, GVHD elicited by Lyt-2⁺ vs. L3T4⁺ cells probably involves different cellular mechanisms. Indeed, we have observed distinct differences in the histopathology of mice with GVHD mediated by Lyt-2⁺ vs. L3T4⁺ cells (to be published). Likewise, other workers (32, 33) have reported separate forms of GVHD in mice injected with class I– vs. class II–disparate cells. As for GVHD, it also seems likely that Lyt-2⁺ and L3T4⁺ cells use different mechanisms to elicit graft rejection. Direct evidence on this question is clearly needed.

Summary

Highly purified populations of C57BL/6 (B6) L3T4⁺ and Lyt-2⁺ T cell subsets were compared for their capacity to exert alloreactivity to class I vs. class II H-2 differences in vivo. B6 Lyt-2⁺ cells responded strongly to the class I–different mutant, bm1, as manifested by (a) DNA synthesis in the spleen of irradiated mice followed by entry of blast cells into thoracic duct lymph, (b) induction of splenomegaly in newborn mice, (c) production of lethal GVHD in irradiated mice, and (d) skin allograft rejection. By all of these parameters, B6 Lyt-2⁺ cells showed almost total unresponsiveness to the class II–different mutant, bm12. Reciprocal results were observed with B6 L3T4⁺ cells, these cells responding strongly against bm12 but not against bm1. In the case of purified T cell subsets from other strains, CBA/Ca and B10.BR L3T4⁺ cells both responded well to a full H-2 difference. Responses by Lyt-2⁺ cells from these strains were weaker, especially for CBA/Ca cells. The implications of these findings are discussed.

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