Induction of Anti-Allo-Class I H-2 Tolerance by Inactivation of CD8+ Helper T Cells, and Reversal of Tolerance through Introduction of Third-Party Helper T Cells

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Summary

The intravenous sensitization of C57BL/6 (B6) mice with class I H-2-disparate B6-C-H-2^{bm1} (bm1) spleen cells resulted in the abrogation of $CD8^+$ T cell–mediated anti-bm1 (proliferative and interleukin 2–producing) T helper (Th) cell activities. In vitro stimulation of lymphoid cells from these mice with bm1 cells, however, generated a reduced, but appreciable, anti-bm1 cytotoxic T lymphocyte (CTL) response. Moreover, the anti-bm1 CTL response, upon stimulation with [bm1 × B6-C-H-2^{bm12} (bm12)]F_1 spleen cells, was enhanced when compared with the response induced upon stimulation with bm1 cells. These in vitro results were reflected on in vivo graft rejection responses; bm1 skin grafts engrafted in the bm1-presensitized B6 mice exhibited prolonged survival, whereas (bm1 × bm12)F_1 grafts placed collateral to bm1 grafts (dual engrafted mice) inhibited the tolerance to bm1. In the B6 mice 1–2 d after rejecting the bm1 grafts, anti-bm1 Th activities remained marginal, whereas potent anti-bm1 CTL responses were found to be generated from their spleen cells. Administration in vivo of anti-CD4 antibody into bm1-presensitized, dual graft–engrafted mice prolonged bm1 graft survival and interfered with enhanced induction of anti-bm1 CTL activity. These results indicate that anti–class I alloantigen (bm1) tolerance as induced by intravenous presensitization with the relevant antigens is not ascribed to the elimination of $CD8^+$ CTL precursors, but to the specific inactivation of $CD8^+$ Th cells, whose function can be bypassed by activating third-party Th cells.

Immune responses to alloantigens are initiated by the recognition of alloantigens by Th cells. Recent studies have revealed the existence of Th subsets that are different in their phenotypes and mode of alloantigen recognition (1–3). Various subsets of Th cells are, thus, considered to initiate anti-allo-immune responses. The overall anti-allo-immune responses consist of a complicated network in which different subsets of T cells, macrophage/APC, and B cells participate. Rosenberg et al. (4) showed that allograft rejection results from interactions especially between T cells of different phenotypes, or of different antigen specificities, and with distinct functions. It would be of importance to consider this when an immune manipulation is attempted to induce the tolerance of anti-allo-immune responses.

The presentation of alloantigens via an intravenous route regulates positively or negatively the induction of anti-allo-immune responses, as has been reported for the effect of donor-specific transfusion (DST)[1] (5–15). Earlier studies from our laboratory have demonstrated that anti-allo-H-2 antigen tolerance, which leads to prolonged graft survival, is inducible by intravenous presensitization with cells expressing the corresponding allo-H-2 antigens (16–18). This was, however, achieved in two simplified donor-host combinations with either class I (16, 17) or class II (18) H-2 disparity. Since engrafting allogeneic tissue with complex disparity induces rejection responses involving complicated cellular interactions, it will be required to investigate how an anti-allo T cell response is influenced by other T cell responses. Such an investigation could contribute to initiating the establishment of anti-allo-tolerance in the donor-host combinations with a complex disparity.

The present study was undertaken to investigate how anti-allo-class I H-2 tolerance is influenced when third-party Th function is introduced by engrafting a class I and class II H-2-disparate graft. The results demonstrate that intravenous presensitization of B6 mice with bm1 cells induced the abrogation of anti-bm1 Th activity and the prolongation of bm1 skin graft survival. However, when bm1-presensitized B6 mice were engrafted with a (bm1 × bm12)F_1 skin graft collateral

[1] *Abbreviations used in this paper:* DST, donor-specific transfusion; FMF, flow microfluorometry; MLR, mixed lymphocyte reaction; SN, supernatant.

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to a bm1 skin graft, the bm1 graft was rejected along with the (bm1 × bm12)F_1 graft without exhibiting its prolonged survival. It is also shown that unprolongation of bm1 graft survival in bm1-presensitized B6 mice engrafted with dual skin grafts is associated with enhanced generation of anti-bm1 CTL activity, and prevented by in vivo injection of anti-CD4 antibody.

Materials and Methods

Mice. C57BL/6 (B6) and BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory. B6-C-H-2^{bm1} (bm1) and B6-C-H-2^{bm12} (bm12) mice were originally provided by D.H. Sachs (National Cancer Institute, Bethesda, MD). These two strains of mutant mice and their hybrid (bm1 × bm12)F_1 mice were bred by mating in our laboratory. These mice were used for experiments at 7–9 wk of age. Female mice were used throughout this study except for donor mice of grafts in some experiments.

Intravenous Presensitization with Allogeneic Cells. Mice were injected with 10^7 allogeneic bm1 spleen cells intravenously.

Media Used for In Vitro Cell Culturing. Culture medium used for the mixed lymphocyte reaction (MLR) was RPMI 1640 supplemented with 10% horse serum, 5×10^{-5} M 2-ME, sodium pyruvate, glutamine, nonessential amino acids, and 5 mM hepes buffer. Complete medium for the generation of CTL was prepared by replacing 10% horse serum with 10% FCS in the above medium. RPMI 1640 supplemented with 5% FCS plus 5×10^{-5} M 2-ME was used for the production of IL-2 and IL-2 assays.

mAbs. Anti-L3T4 (GK1.5) (19) and anti-Lyt-2 (3.155) (American Type Culture Collection, Rockville, MD) mAbs were obtained from culture supernatants of hybridomas producing the relevant antibody. GK1.5 mAb was also obtained in a form of ascitic fluid. Gamma globulin fraction of the ascitic fluid was obtained by precipitation at 50% saturation with ammonium sulfate and used for in vivo administration.

Treatment of Lymphoid Cells with Antibody Plus Complement (C). Spleen or lymph node cells (10^8) were incubated at 4°C for 30 min with appropriate dilutions of anti-L3T4 or -Lyt-2 mAb. Cells were washed and incubated at 37°C for 45 min with rabbit C preabsorbed with syngeneic mouse spleen cells at a final dilution of 1:20. The efficacy of these antibody treatments was confirmed in flow microfluorometric analysis by demonstrating that the treatment with anti-L3T4 or -Lyt-2 antibody results in almost complete elimination of the respective Lyt-2^+ ($CD8^+$) or L3T4^+ ($CD4^+$) T cell subset without damage onto the other alternative T cell subset.

MLR. The responding lymph node or spleen cells, obtained from a pool of three to five animals per group, were resuspended in complete medium and cultured at a concentration of 2×10^5 cells per culture well together with 4×10^5 irradiated (2,000 rad) stimulating spleen cells in a total volume of 0.2 ml (16–18). Each MLR was performed in triplicate in flat-bottomed microculture plates (25860; Corning Glass Works, Corning, NY) and maintained in a humidified atmosphere at 5% CO_2 at 37°C. The cultures were harvested at 96 h after an 8-h pulse with 20 KBq/well of [^{3}H]TdR by using an automatic cell harvester onto glass fiber filters. Radioactivity was determined by liquid scintillation spectrometry. Results were calculated from uptake of [^{3}H]TdR and expressed as the mean uptake in cpm ± SE of triplicate cultures.

Generation of CTL. The CTL induction in macrocultures was performed as previously described (20). Briefly, 5×10^6 lymph node or spleen cells were cultured in vitro with 10^6 irradiated (2,000 rad) syngeneic or allogeneic spleen cells in 24-well culture plates (25820; Corning Glass Works) in a volume of 2 ml at 37°C for 5 d. Effector cells generated were assayed on ^{51}Cr-labeled target cells (spleen cells cultured for 48 h with Con A, 5 μg/ml). Percent specific lysis was calculated as described (20). SEs in each group were < 10%, and these were excluded from the figures for simplicity. To detect limiting effects of CTL precursors, CTL induction was performed in some experiments according to limiting dilution assay as described (17). Briefly, graded numbers of lymph node cells were cultured with 10^5 x-irradiated stimulating spleen cells in 96-well microplates in the absence of exogenous IL-2, and 5 d later, ^{51}Cr-labeled Con A blast target cells (10^4) were directly added to each culture well (six wells/group).

Production of IL-2 and Assay System for IL-2 Activity. A mixture of lymph node and spleen cells (5×10^6) was cultured with 3×10^6 syngeneic or allogeneic stimulator spleen cells (2,000 rad) in 24-well culture plates (25820; Corning Glass Works) in a volume of 1 ml (16–18). After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants (SN) were harvested by centrifugation and stored at −20°C until use.

SN were assayed for IL-2 activity according to their ability to support the proliferation of the IL-2-dependent T cell line CTLL-2 (16–18). CTLL-2 (10^4/well) were cultured with the supernatant in a volume of 0.2 ml in 96-well flat-bottomed microplates (25860; Corning Glass Works) for 24 h at 37°C. Proliferation was assessed by the uptake of [^{3}H]TdR during a 4-h pulsing with 20 KBq [^{3}H]TdR/well.

FITC-conjugated Antibodies. FITC was conjugated to GK1.5 mAb by regular methods, followed by DE-52 (Whatman Biochemicals Ltd., England) ion exchange chromatography (21). The F/P ratio of FITC/GK1.5 was 1.6. FITC-conjugated anti-Lyt-2 and mouse anti-rat Ig antibodies were the products of Becton Dickinson Immunocytometry Systems, Mountain View, CA.

Immunofluorescence Staining and Flow Microfluorometry (FMF). The cell preparation and staining procedures were essentially the same as described previously (22). Briefly, 10^6 lymphoid cells were incubated at 4°C for 30 min with FITC-conjugated antibodies, washed twice, resuspended, and analyzed for fluorescence. These procedures were performed in HBSS (without phenol red) containing 0.1% BSA and 0.1% sodium azide. FMF analysis was performed by using a FACStar (Becton Dickinson Immunocytometry Systems). All data were collected by using log amplification, and dead cells were rejected from analysis by additional staining with propidium iodide.

Skin Grafting of B6 Mice. B6 mice were engrafted on the flank with two tail skin grafts, separated by a host skin bridge, according to an adaptation of the method of Billingham and Medawar (23). Bandages were removed on day 7, and the grafts were scored daily until rejection (defined as loss of > 80% of the grafted tissue).

Results

Responses of Lymphoid Cells from bm1-presensitized B6 Mice to bm1 Alloantigens upon the Stimulation with (bm1 × bm12)F_1 Cells. B6 mice were injected intravenously with 10^7 bm1 spleen cells. 1 wk later, lymph node cells were obtained from these bm1-presensitized or -unpresensitized B6 mice and treated with anti-CD4 (GK1.5) or anti-CD8 (3.155) mAb plus C. The resultant cells ($CD8^+$ or $CD4^+$ subset of T cells) or unfractionated cells were stimulated in vitro with irradiated bm1 or (bm1 × bm12) cells. The results of Tables 1 and 2 confirm the fact (16, 17) that intravenous presensitization of B6 mice

Table 1. *Phenotype of Anti-(bm1 × bm12)F_1-reactive Proliferating T Cells in B6 Mice Presensitized with bm1 Spleen Cells*

Responding cells		[^{3}H]TdR Uptake after stimulation with:§		
B6 mice presensitized with:*	Treatment with:‡	B6	bm1	(bm1 × bm12)F_1
			cpm × 10^{-3}	
B6	C	2.5 ± 0.2	29.4 ± 0.1	54.3 ± 1.6
	Anti-CD4 + C	ND	22.7 ± 1.4	28.5 ± 2.6
	Anti-CD8 + C	ND	2.9 ± 0.5	24.1 ± 4.3
bm1	C	3.3 ± 0.5	5.4 ± 1.0	22.4 ± 3.8
	Anti-CD4 + C	ND	3.5 ± 0.7	2.9 ± 0.4
	Anti-CD8 + C	ND	2.4 ± 0.3	24.2 ± 2.2

* B6 mice were injected intravenously with 10^7 bm1 spleen cells.
‡ Lymph node cells 1 wk after the bm1 presensitization were treated with anti-CD4 (GK1.5) or anti-CD8 (3.155) antibody plus C.
§ Lymph node cells (2×10^5/well) were cultured with 2,000-rad X-irradiated spleen cells (4×10^5/well) from the indicated strains of mice for 4 d.

with bm1 alloantigens resulted in almost complete abrogation of CD8$^+$ T cell–mediated anti-bm1 proliferative (Table 1) and IL-2-producing (Table 2) capacities. It is also shown that since the presensitization with bm1 cells did not affect the responsiveness of CD4$^+$ T cell subset, this subset of T cells exhibited comparable magnitudes of anti-bm12 responses upon the stimulation with (bm1 × bm12)F_1 cells in both MLR and IL-2 production.

Earlier studies from our laboratory have also demonstrated that intravenous presensitization with bm1 cells failed to abrogate CD8$^+$ T cell–mediated anti-bm1 CTL responses in contrast to the elimination of CD8$^+$ Th (proliferative and IL-2-producing) capacities (16, 17). The results of Fig. 1 confirm that potent anti-bm1 CTL responses are generated in bulk cultures of spleen cells from bm1-presensitized mice (4×10^5/well). When the number of responding spleen cells was reduced ($2-1 \times 10^5$/well), the activity of anti-bm1 CTL was appreciably or considerably weaker compared with that obtained by using the same number of normal B6 responding cells. The results of Fig. 1 also show that in cultures at the reduced responding cell numbers, cells from bm1-presensitized mice induced slightly enhanced generation of anti-bm1 CTL responses upon the stimulation with (bm1 × bm12)F_1 cells when compared with responses induced upon stimulation with bm1 cells, and that such an enhanced generation of anti-bm1 CTL was abrogated by elimination of

Table 2. *Phenotype of Anti-(bm1 × bm12)F_1-reactive IL-2-producing T Cells in B6 Mice Presensitized with bm1 Spleen Cells*

Responding cells*		[^{3}H]TdR Uptake in IL-2 production after stimulation with:‡		
B6 mice presensitized with:	Treatment with:	B6	bm1	(bm1 × bm12)F_1
			cpm × 10^{-3}	
B6	C	0.9 ± 0.1	11.7 ± 2.1	21.9 ± 1.8
	Anti-CD4 + C	ND	12.9 ± 0.3	7.9 ± 0.1
	Anti-CD8 + C	ND	2.1 ± 0.1	24.3 ± 0.7
bm1	C	0.5 ± 0.1	0.9 ± 0.1	20.3 ± 1.2
	Anti-CD4 + C	ND	0.9 ± 0.1	0.7 ± 0.1
	Anti-CD8 + C	ND	1.8 ± 0.1	22.8 ± 3.2

* Responding cells were the same as used in Table 1.
‡ Responding cells (5×10^6/well) were cultured with 2,000-rad X-irradiated spleen cells (3×10^6/well) from the indicated strains of mice for 2 d. Culture supernatants were submitted to IL-2 assay using CTLL-2 cells.

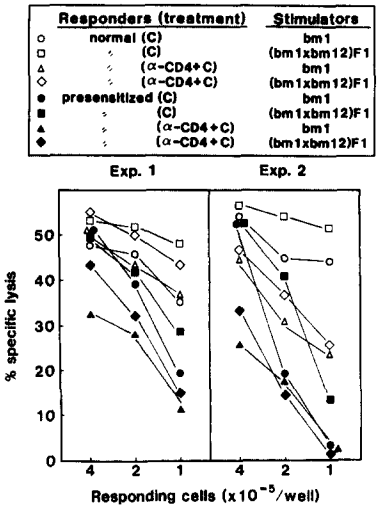

Figure 1. Anti-bm1 CTL potentials in bm1-presensitized B6 mice. Lymph node cells from normal or bm1-presensitized B6 mice were untreated or treated with anti-CD4 antibody plus C. Graded numbers of these cells were cultured with 10^5 stimulating cells (indicated) in 96-well microplates. Anti-bm1 CTL activity was assessed by adding ^{51}Cr-labeled bm1 blast target cells directly into each well. The results are shown as the mean percent specific lysis of sextuplicate cultures/group.

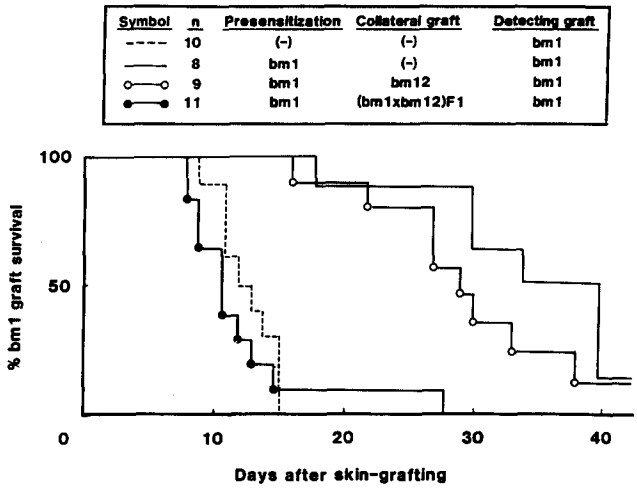

Figure 2. (bm1 × bm12)F_1 collateral skin grafting induces the rejection of bm1 skin graft in bm1-presensitized B6 mice. B6 mice 1 wk after bm1 presensitization were engrafted with a detecting bm1 skin graft on the right flank and the indicated collateral skin graft on the left flank.

$CD4^+$ T cells from responding cells before CTL induction. These results suggest that the anti-bm1 CTL responses of cells from the bm1-presensitized B6 mice are reduced due to elimination of anti-bm1 $CD8^+$ Th cells, but these responses may be augmented by introduction of an alternative $CD4^+$ Th mechanism (anti-bm12 Th).

Anti-bm1 Graft-rejecting Capacity Reduced by Intravenous Presensitization Is Recovered by Engrafting (bm1 × bm12)F_1 Collateral Grafts. B6 mice were presensitized intravenously with bm1 cells, and 1 wk later, these bm1-presensitized mice were engrafted with a bm1 graft (detecting graft) on the right flank along with a collateral graft [bm12 or (bm1 × bm12)F_1] on the left flank. The results of Fig. 2 illustrate that (*a*) intravenous presensitization of B6 mice with bm1 cells results in the prolonged survival of bm1 skin grafts, which confirms the previous report (17); and (*b*) such prolongation of bm1 graft survival is prevented when B6 recipients are engrafted with a (bm1 × bm12)F_1 skin graft as a collateral graft. It should be noted that engrafting bm12 skin grafts did not prevent the prolongation of the bm1 skin graft survival, which is induced by intravenous presensitization with bm1 cells.

Analysis of Anti-bm1 T Cell Responses in bm1-presensitized B6 Mice after (bm1 × bm12)F_1 Skin Grafting. To determine cellular mechanisms by which the prolongation of bm1 skin graft survival in bm1-presensitized B6 mice is prevented by engrafting (bm1 × bm12)F_1 collateral grafts, we examined anti-bm1 Th (proliferative and IL-2-producing) (Tables 3 and 4), as well as CTL, activities (Fig. 3) in such B6 mice. B6 mice were presensitized with bm1 cells and engrafted with bm1 skin grafts along with bm12 or (bm1 × bm12)F_1 skin grafts. The results demonstrate that lymphoid cells from bm1-presensitized mice 10 d after the dual skin grafting exhibit only weak anti-bm1 proliferative (Table 3) and only marginal IL-2-producing (Table 4) activities. These results indicate that potent anti-bm1 suppressive state is still maintained at the $CD8^+$ Th level, even after the dual skin-grafting.

Fig. 3 shows the generation of CTL activity by spleen cells obtained from the same mice as used in Tables 3 and 4. Appreciable levels of anti-bm1 CTL responses generated in B6 mice receiving only bm1 presensitization regimen were attenuated after bm1 skin grafting (Fig. 3), which is consistent with the previous results (17). Such a reduced anti-bm1 CTL potential was also observed in bm1-presensitized B6 mice that were engrafted with a bm12 collateral skin graft (Fig. 3). However, when bm1-presensitized mice were engrafted with a (bm1 × bm12)F_1 graft as a collateral graft along with a bm1 skin graft, spleen cells from these mice (10 d after the dual skin grafting) produced potent anti-bm1 CTL activity. Such an activity was comparable with or slightly higher than that observed in unpresensitized normal B6 mice. These results indicate that anti-bm1 CTL potential in bm1-presensitized mice, which is otherwise attenuated by bm1 skin grafting, can be induced for its enhanced activation when the recipient mice are engrafted with (bm1 × bm12)F_1 collateral grafts.

Requirement of $CD4^+$ T Subset for Preventing the Prolongation of bm1 Skin Graft Survival in bm1-presensitized, Dual Skin-engrafted B6 Mice. We next investigated whether $CD4^+$ T cells reactive with bm12 alloantigens are involved in the prevention of prolonged bm1 graft survival in bm1-presensitized mice receiving a collateral (bm1 × bm12)F_1 graft. Semipurified anti-CD4 (GK1.5) antibody was administered in vivo at the dose of 100 μg/d, three times/wk to eliminate $CD4^+$ T cells. This procedure was continued for 1–8 wk, and at each stage, the efficacy of the procedure to deplete $CD4^+$ T cells was examined. Intraperitoneal injection of anti-CD4 an-

Table 3. *Anti-bm1-proliferating T Cell Activity in bm1-presensitized B6 Mice 10 d after Dual Skin Grafting*

B6 mice presensitized with:	Responding cells*		[^{3}H]TdR Uptake after stimulation with:‡		
	Detecting graft	Collateral graft	B6	bm1	BALB/c
				cpm × 10^{-3}	
–	–	–	1.6 ± 0.4	24.9 ± 1.5	25.9 ± 1.6
bm1 (pooled)	bm1	–	2.0 ± 0.3	3.3 ± 0.5	26.2 ± 2.1
bm1 1	bm1	bm12	2.2 ± 0.4	6.3 ± 0.8	26.0 ± 3.9
2	bm1	bm12	2.7 ± 0.6	7.4 ± 0.4	34.1 ± 2.0
3	bm1	bm12	1.6 ± 0.1	3.6 ± 0.5	23.8 ± 2.2
bm1 1	bm1	(bm1 × bm12)F_1	2.2 ± 0.8	5.9 ± 0.6	28.8 ± 3.4
2	bm1	(bm1 × bm12)F_1	2.2 ± 0.2	5.9 ± 0.5	15.3 ± 1.4
3	bm1	(bm1 × bm12)F_1	2.8 ± 0.5	9.1 ± 1.2	27.2 ± 1.9
4	bm1	(bm1 × bm12)F_1	2.5 ± 0.1	4.3 ± 0.9	20.1 ± 1.0

* bm1-presensitized B6 mice were engrafted with a bm1-detecting graft and the indicated collateral graft.
‡ 10 d after the dual skin grafting, lymph node cells were harvested and stimulated with spleen cells (shown) in MLR.

tibody for 1 wk (three times) induced the depletion of > 90% CD4$^+$ T cells (data not shown). Administration of the antibody for an additional week (2 wk, six times) resulted in almost complete elimination of CD4$^+$ T cells without affecting the CD8$^+$ T cell subset (Fig. 4). The CD4$^+$ T cell–depleted state was maintained by continued administration of the anti-CD4 antibody for 8 wk (data not shown).

bm1-presensitized B6 mice were engrafted with bm1 and bm12 or (bm1 × bm12)F_1 collateral skin grafts, and the above protocol of anti-CD4 antibody administration was started on the same day as the dual skin grafting (Fig. 5). The results illustrate that in vivo administration of anti-CD4 antibody resulted in an appreciable prolongation of bm1 skin graft survival under conditions in which bm1-presensitized B6 mice were engrafted with bm1 and (bm1 × bm12)F_1 skin grafts. These results indicate that the CD4$^+$ T cell subset is responsible for preventing the prolongation of bm1 graft survival in the dual grafting model.

Table 4. *Anti-bm1 IL-2-producing T Cell Activity in bm1-presensitized B6 Mice 10 d after Dual Skin Grafting*

B6 mice presensitized with:	Responding cells*		IL-2 production after stimulation with:		
	Detecting graft	Collateral graft	B6	bm1	BALB/c
				cpm × 10^{-3}	
–	–	–	1.7 ± 0.5	22.9 ± 2.1	17.1 ± 2.7
bm1 (pooled)	bm1	–	1.1 ± 0.3	2.4 ± 0.2	16.7 ± 1.5
bm1 1	bm1	bm12	1.2 ± 0.2	1.4 ± 0.1	14.3 ± 1.9
2	bm1	bm12	1.3 ± 0.4	1.1 ± 0.1	10.1 ± 1.6
3	bm1	bm12	1.2 ± 0.1	1.3 ± 0.1	14.4 ± 0.8
bm1 1	bm1	(bm1 × bm12)F_1	1.3 ± 0.0	1.3 ± 0.2	15.6 ± 0.8
2	bm1	(bm1 × bm12)F_1	0.6 ± 0.1	0.8 ± 0.1	13.8 ± 1.2
3	bm1	(bm1 × bm12)F_1	1.9 ± 0.3	3.2 ± 0.6	18.0 ± 1.9
4	bm1	(bm1 × bm12)F_1	1.4 ± 0.1	1.8 ± 0.2	15.3 ± 2.0

* Responding spleen cells were obtained from the same donor mice as used in Table 3.

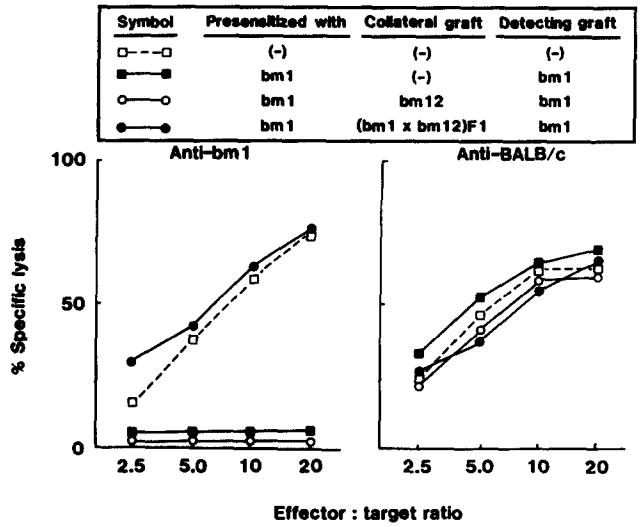

Figure 3. Generation of anti-bm1 CTL responses in bm1-presensitized B6 mice 10 d after dual skin grafting. A source of responding spleen cells was the same as used in Table IV. Each group of these cells was pooled and stimulated in vitro with bm1 or the third party of BALB/c spleen cells and assayed on bm1 or BALB/c (control) blast target cells.

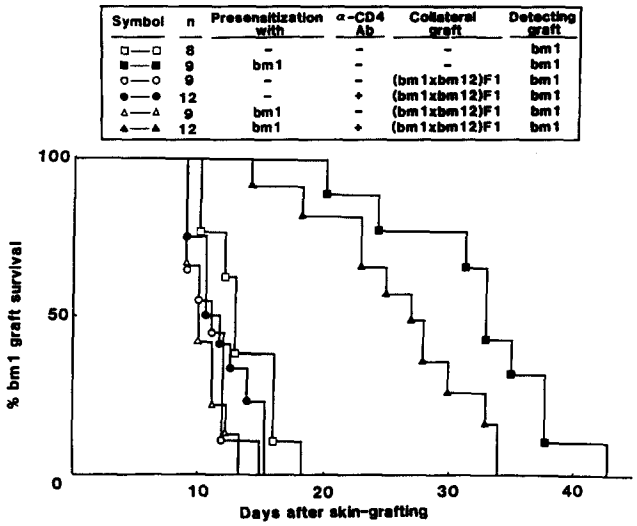

Figure 5. Effect of (bm1 × bm12)F_1 skin grafting is prevented by in vivo administration of anti-CD4 antibody. B6 mice were unpresensitized or presensitized with bm1 cells and engrafted with two skin grafts as indicated. The injection of anti-CD4 antibody (100 μg/day, three times/wk) was started on the same day as the dual skin grafting.

Additional experiments were performed to investigate whether the in vivo effect of anti-CD4 antibody administration is related to the suppression of anti-bm1 CTL generation. bm1-presensitized B6 mice were engrafted with two skin grafts [bm1 and (bm1 × bm12)F_1], and the administration of anti-CD4 antibody was started simultaneously with the dual skin grafting. 10 d after the dual skin grafting, spleen cells from these antibody-treated or untreated mice were tested for their ability to generate anti-bm1 CTL responses. The results of Fig. 6 demonstrate that administration in vivo of anti-CD4 antibody prevents the generation of anti-bm1 CTL responses that are otherwise induced by spleen cells from bm1-presensitized B6 mice after two skin [bm1 and (bm1 × bm12)F_1] graftings. Taken collectively, these results indicate that elimination of the $CD4^+$ T cell subset results in the prevention of the prolonged bm1 graft survival along with inhibition of the generation of anti-bm1 CTL responses.

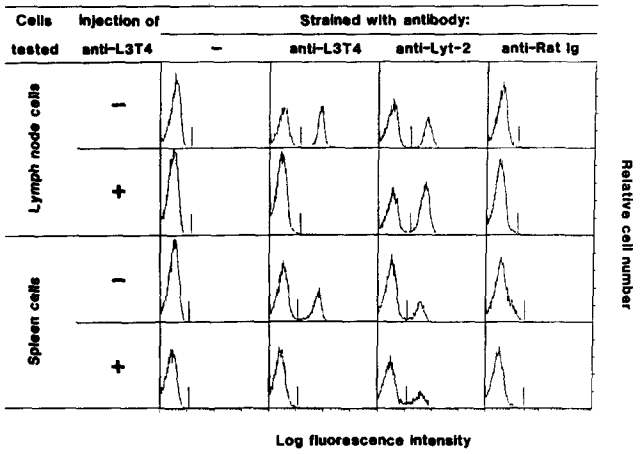

Figure 4. Depletion of $CD4^+$ T cells by in vivo administration of anti-CD4 antibody. Semi-purified anti-CD4 (GK1.5) antibody was injected intravenously at the dose of 100 μg/day three times per week for 2 wk. Lymph node and spleen cells from these antibody-treated mice were submitted to FMF analyses.

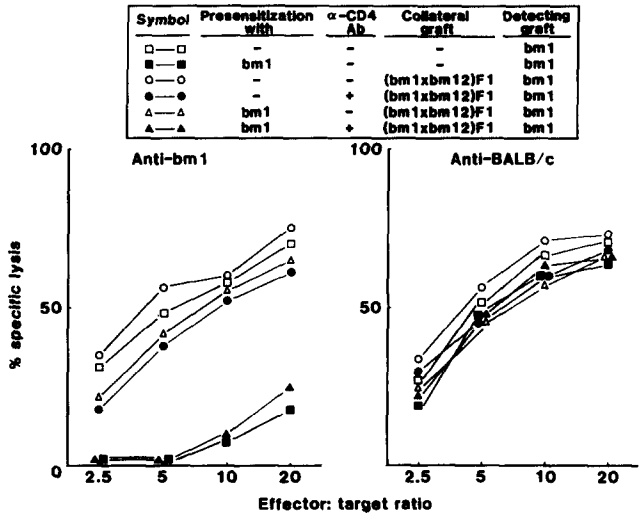

Figure 6. Suppression of anti-bm1 CTL generation in bm1-presensitized, dual skin-engrafted B6 mice by injection of anti-CD4 antibody. B6 mice were unpresensitized or presensitized with bm1 cells and engrafted with the indicated detecting and collateral grafts. The injection of anti-CD4 antibody was started on the same day as the dual skin grafting. Spleen cells from each group of mice were obtained 10 d later.

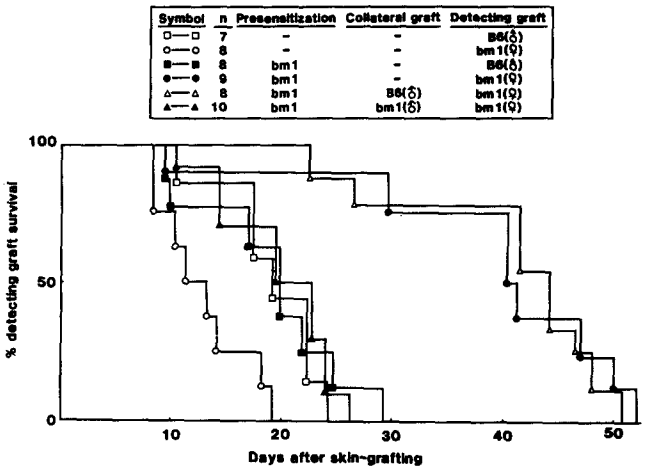

Figure 7. Male bm1 collateral skin grafting induces the rejection of female bm1 grafts in bm1-presensitized female mice. Unpresensitized or bm1-presensitized female B6 mice were engrafted with the indicated collateral and detecting grafts.

Breakdown of Anti-bm1 CD8⁺ T Cell Tolerance by Introduction of anti-male Th Mechanism. We finally investigated whether breakdown of anti-bm1 CD8⁺ T cell tolerance by the introduction of an alternative Th mechanism can be generalized, i.e., breakdown of this tolerance is inducible by introducing on a collateral (sensitizing) graft other cell surface antigens than H-2 antigens. Female B6 mice were presensitized with female bm1 cells, as has been performed in the preceding experiments. These bm1-presensitized and unpresensitized B6 mice were engrafted with one or two skin grafts that were of B6 or bm1, and male or female. The results are summarized in Fig. 7. The results demonstrate that (*a*) B6 male grafts were rejected in B6 female recipients although the rejection was significantly delayed compared with that of allogeneic bm1 (female) grafts; (*b*) the survival of female bm1-detecting grafts was prolonged in bm1-presensitized mice. This was also the case when these recipient mice were engrafted with male B6 collateral grafts that themselves were rejected in comparable time courses with those observed for male B6-detecting grafts; (*c*) in contrast, engrafting male bm1-collateral grafts resulted in appreciably accelerated rejection of female bm1-detecting grafts. These results indicate that (*a*) cell-cell interactions capable of mounting enhanced rejection responses are observed between T cells against various types of antigens; and (*b*) coexpression of these antigens on a single graft is required for the induction of enhanced rejection responses.

Discussion

A major issue in transplantation immunology is to analyze the mechanisms underlying cellular interactions involved in in vivo responses to alloantigens. It has been shown that allograft rejection results from interactions between Th and effector T cells (e.g., CTL) of different phenotypes and of different antigen specificities (4), although rejection of class I H-2-disparate grafts can be mediated by isolated CD8⁺ T cell populations (24, 25).

Another important goal is the establishment of immune manipulations by which clonal elimination and/or functional inactivation of alloantigen-reactive T cells are achieved. One of immunobiological manipulations would be to attempt negative regulation of immune responses by introduction of alloantigens via an intravenous route. This effect has been recognized as the beneficial one of pretransplant DST in clinical studies (5–10). We have also demonstrated in animals studies that CD8⁺ T cell responses to allo-class I H-2 or CD4⁺ T cell responses to allo-class II H-2 antigens can be tolerized by intravenous presensitization with cells expressing the corresponding alloantigens (16–18). In considering that cellular interactions between phenotypically and functionally distinct subsets of T cells are involved in the rejection responses in general, it would also be important to investigate how each component of T cell responses is mutually influenced by other component(s) of these.

In the present study, we have investigated the effects of cellular interactions on the induction and/or maintenance of anti-allo-tolerance induced by the above-mentioned intravenous presensitization regimen. The results demonstrate that (*a*) intravenous presensitization of B6 mice with class I H-2-disparate bm1 cells results in the abrogation of CD8⁺ Th cell activity, leading to the prolongation of bm1 graft survival; (*b*) when bm1-presensitized mice were engrafted with bm1 and (bm1 × bm12)F_1 grafts, the survival of the bm1 graft was not prolonged, but this graft was rejected along with the (bm1 × bm12)F_1 graft; (*c*) only marginal or potent anti-bm1 CTL activity was generated by spleen cells from bm1-presensitized mice that were followed by engrafting only bm1 or two [bm1 and (bm1 × bm12)F_1] grafts, respectively; (*d*) the effect of engrafting collateral (bm1 × bm12)F_1 grafts on the generation of CTL responses and inhibition of prolonged bm1 graft survival was abrogated by eliminating CD4⁺ T cells able to react with bm12 alloantigens and to exert an alternative (anti-bm12) Th activity; and (*e*) a similar effect of cell-cell interaction to that observed between anti-bm1 (allo-class I H-2) and anti-bm12 (allo-class II H-2) T cells was also inducible by the interaction between anti-bm1 and anti-male (non-H-2) responses.

The intravenous presensitization with bm1 cells did not eliminate the total anti-bm1 CTL capacity. The reduced but appreciable frequencies of CTL precursors were detected in lymph node and spleen cells (17). Such CTL potential remaining in the bm1-presensitized mice was suppressed along with the engrafting of bm1 skin grafts (17). The present results that bm1 presensitized, two graft [bm1 and (bm1 × bm12)F_1]-engrafted mice were capable of developing enhanced anti-bm1 CTL responses indicated the involvement of anti-bm12 responses in the generation of potent anti-bm1 CTL responses. Thus, it is conceivable that CD4⁺ T cell-mediated anti-bm12 Th cell activity helped to activate anti-bm1 CTL precursors remaining in the bm1-presensitized mice that are otherwise attenuated after the engrafting of bm1 grafts alone. It is also possible that such CTL activity contributed to the rejection in vivo of bm1 grafts. The results that elimi-

nation of CD4+ T cells by administrating anti-CD4 antibody fails to generate these in vitro and in vivo effects could support the above postulation.

Cell-cell interactions similar to those observed here were originally reported by Rosenberg et al. (4). In their model, CD4+ Th cells reactive to allo-class II H-2 generated help for the activation of anti-K^{bm6} or $Qa1^a$ CTL precursors that represent helper-dependent effectors and fail to mature without help from Th cells against other alloantigens. Thus, their observations and ours illustrate the role of CD4+ Th cells in positively regulating the activation of CTL responses to other alloantigens in various combinations.

The present study should be discussed from two distinct aspects. First, our results indicated that the activation of anti-bm12 CD4+ Th cells is not sufficient for enhanced induction of anti-bm1 CTL, as well as prevention of the prolonged bm1 graft survival, since the above in vitro and in vivo effects were induced when the recipient mice receiving bm1 grafts were engrafted with (bm1 × bm12)F_1 but not with bm12 grafts as collateral ones. Thus, an appropriate microenvironment could be required under which the help of anti-bm12 CD4+ Th cells functions to efficiently activate anti-bm1 CTL precursors. It is conceivable that (bm1 × bm12)F_1 cells expressing both bm1 and bm12 alloantigens are responsible for this cell-cell interaction process. It has been established that the stimulation of Th cells, especially priming of virgin Th cells, requires the participation of APC expressing helper determinants (1, 2). Therefore, in this model, (bm1 × bm12)F_1 cells that express helper (bm12) determinants and bm1 CTL antigens, and possess APC functions, i.e., Ia+ cells such as Langerhans cells, might be visualized as operating for the cellular interaction. It may also be possible that once anti-bm12 Th cells are activated to produce IFN-γ capable of inducing the expression of Ia antigens on Ia− cells (26–28), skin cells rendered Ia+ by IFN-γ (keratinocytes) might provide the function for stimulating both Th cells and CTL.

Another important aspect is concerned with the physiologic relevance of the present study. Since DST is attempted by using cells from the donor that provides the graft, the situation made in the present study, i.e., the activation of anti-class I CTL by anti-class II CD4+ Th cells, would be unrealistic. Nevertheless, it would be of value to consider the clinical relevance of the present observations based on cellular interactions. Although only described for skin (29), the existence of tissue- or organ-specific histocompatibility antigens has been suggested (30). It could be that some of such tissue- or organ-specific histocompatibility antigens function as helper determinants, as has been observed for bm12 alloantigens. Thus, the existence of tissue- or organ-specific histocompatibility antigens is of practical importance when the induction of donor-specific tolerance attempts to use cells of one tissue type, but organs of another tissue type are transplanted. The fact that the coexpression on a graft of male-specific antigen as a model of non-H-2 antigen induced enhanced rejection responses could support this notion.

The present results illustrate that the induction and/or maintenance of tolerance to a given alloantigen is influenced by cellular interactions between T cells against that antigen and other antigens coexpressed on a graft. Our recent study (Hori, S., S. Kitagawa, S. Sato, T. Hamaoka, and H. Fujiwara, manuscript in preparation) also revealed that the effect of intravenous presensitization is modulated by cellular interactions between anti–class I and –class II H-2 T cells when class I– and class II–disparate cells are used for the intravenous presensitization. Taken together, these approaches could contribute to a better understanding of complex cellular interactions involved in graft rejection responses in vivo, and provide the validity for attempting to apply such studies to clinical transplantation immunology.

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