Heterogenous Graft Rejection Pathways in Class I Major Histocompatibility Complex-disparate Combinations and Their Differential Susceptibility to Immunomodulation Induced by Intravenous Presensitization with Relevant Alloantigens

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Summary

The present study investigates the heterogeneity of graft rejection pathways in class I major histocompatibility complex (MHC)-disparate combinations and the susceptibility of each pathway to immunomodulation induced by intravenous presensitization with alloantigens. Depletion of $CD8^+$ T cells was induced by repeated administration of anti-CD8 monoclonal antibody. $CD8^+$ T cell-depleted mice failed to generate anti-allo class I MHC cytotoxic T cell (CTL) responses but exhibited anti-allo class I MHC T cell responses, such as mixed lymphocyte reaction (MLR)/IL-2 production, that were induced by $CD4^+$ T cells. In contrast, donor-specific intravenous presensitization (DSP), as a model of donor-specific transfusion, induced almost complete elimination of $CD4^+$ and $CD8^+$ T cell-mediated MLR/IL-2 production, whereas this regimen did not affect the generation of CTL responses induced by DSP-resistant elements ($CD8^+$ CTL precursors and $CD4^+$ CTL helpers). Prolongation of skin graft survival was not induced by either of the above two regimens alone, but by the combination of these. Prolonged graft survival was obtained irrespective of whether the administration of anti-CD8 antibody capable of eliminating CTL was started before or after DSP. The combination of DSP with injection of anti-CD4 antibody also effectively prolonged graft survival. However, this was the case only when the injection of antibody was started before DSP, because such antibody administration was capable of inhibiting the generation of CTL responses by eliminating DSP-resistant $CD4^+$ CTL helpers. These results indicate that (*a*) the graft rejection in class I-disparate combinations is induced by $CD8^+$ CTL-involved and -independent pathways that are resistant and susceptible to DSP, respectively; (*b*) DSP contributes to, but is not sufficient for, the prolongation of graft survival; and (*c*) the suppression of graft rejection requires an additional treatment for reducing DSP-resistant CTL responses. The results are discussed in the context of potential clinical application in attempts to inhibit the generation of DSP-resistant CTL responses upon the prospective DSP.

Immune responses to alloantigens are initiated by the recognition of alloantigens by T helper (Th) cells. These Th cells consist of multiple subsets that differ from one another in their phenotype and mode of alloantigen recognition (1–3). It has been shown that various subsets of Th cells initiate anti-allo-immune responses, including CTL responses, and that allograft rejection results from complicated cellular interactions, especially between T cells of different phenotypes, of different antigen-specificities, and with distinct functions (4).

The presentation of alloantigens via the intravenous route positively or negatively regulates 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 several laboratories have demonstrated that the reduction of alloreactivity, as monitored by graft rejection responses, is inducible by intravenous presensitization with cells expressing the corresponding donor alloantigens (donor-specific intravenous presensitization [DSP]) as a model of DST (16–20). This was, however, achieved in simplified donor-host combinations, for example, in a donor-host combination differing

[1] *Abbreviations used in this paper:* DSP, donor-specific intravenous presensitization; DST, donor-specific transfusion; FMF, flow microfluorometry; SN, supernatants.

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only by a mutation in class I MHC (16–19). In contrast, the DSP in conventional class I MHC disparity failed to produce a beneficial effect on subsequent allograft survival (21). To establish the mechanistic basis for the pretransplant DST/DSP effect, as well as to facilitate clinical application of this model, it remains to be investigated which element(s) of the immune response determines the difference in outcomes after DSP in two types of class I MHC disparity (simple mutation vs. conventional disparity). Such an investigation could provide an approach to abrogating the DSP-resistant elements of anti-allo-immune responses.

The present study investigated the heterogeneity of graft rejection pathways induced by conventional class I MHC disparity and the DSP susceptibility of each pathway and/or element of immune response. The results demonstrate three major conclusions: First, the graft rejection in class I–disparate combinations is induced by: (*a*) a $CD8^+$ CTL-involved pathway consisting of $CD8^+$ CTL precursors and allo-class I MHC-restricted $CD8^+$ Th cells or allo-class I MHC-reactive, self class II–restricted $CD4^+$ Th cells; and/or (*b*) $CD8^+$ CTL-independent pathway(s) as detected by MLR/IL-2 production. Second, the CTL-involved pathway generated by CTL precursors and allo-class I MHC-reactive, self class II–restricted $CD4^+$ Th cells is resistant to DSP, whereas the CTL-independent pathway is susceptible to DSP. Third, the prolongation of class I–disparate graft survival is achieved by the combination of DSP with additional treatment to reduce the generation of CTL by eliminating CTL or CTL helpers.

Materials and Methods

Mice. Mouse strains used in this study are shown in Table 1. C57BL/6 (B6), B10.BR, and B10.A mice were purchased from Shizuoka Experimental Animal Laboratory. B6.C-H-2^{bm1} (bm1), B10.AKM, and B10.QBR mice were originally provided by Dr. David Sachs (National Cancer Institute, Bethesda, MD) and bred in our laboratory.

Intravenous Presensitization with Allogeneic Cells (Donor-specific Presensitization). Mice were injected with 10^7 allogeneic spleen cells via the intravenous route.

Media Used for In Vitro Cell Culturing. Culture medium used for the 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.

Monoclonal Antibodies. Anti-CD4 (GK1.5) (22) and anti-CD8 (2.43) (American Type Culture Collection, Rockville, MD) mAbs were obtained from ascitic fluids of hybridomas producing the relevant antibody. Gammaglobulin fraction of the ascitic fluid was obtained by precipitation at 50% saturation with ammonium sulfate.

Mixed Lymphocyte Reaction. The responding lymph node cells, obtained from a pool of three 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, 20). Each MLR was performed in triplicate in round-bottomed microculture plates (25850; Corning Glass Works, Corning, NY) and maintained in 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 Cytotoxic T Lymphocytes. The CTL induction was performed as previously described (23). Briefly, 5×10^6 lymph node or spleen cells were cultured in vitro with $4–10 \times 10^5$ 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 at 5 μg/ml). Percent specific lysis was calculated as described (23). SEs in each group were <10%, and these were excluded from data for simplicity.

Production of IL-2 and Assay System for IL-2 Activity. Lymph node cells (2×10^6/well) were cultured with 4×10^6 syngeneic or allogeneic stimulator spleen cells (2,000 rad) in 24-well culture plates (25820; Corning Glass Works) in a volume of 2 ml (16, 18, 20). After incubation at 37°C in a humidified CO_2 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, 20). 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 4-h pulsing with 20 KBq [^{3}H]TdR/well.

Immunofluorescence Staining and Flow Microfluorometry (FMF). The cell preparation and staining procedures were essentially the same as described previously (24). 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, Mountain View, CA). 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. Mice were engrafted on the flank with tail skin grafts according to an adaptation of the method of Billingham and Medawar (25). Bandages were removed on day 7 and the

Table 1. *H-2 Haplotypes of Mouse Strains Used in This Study*

	H-2 region			
Strain	K	I-A	I-E	D
C57BL/6 (B6)	b	b	–	b
B6.C-H-2^{bm1} (bm1)	bm1	b	–	b
B6.C-H-2^{bm12} (bm12)	b	bm12	–	b
B10.BR	k	k	k	k
B10.A	k	k	k	d
B10.AKM	k	k	k	q
B10.QBR	b	b	–	q

grafts were scored daily until rejection (defined as loss of >80% of the grafted tissue).

Depletion of CD8⁺ or CD4⁺ T Cells. The procedure was the same as described (21, 24). Semipurified anti-CD8 or anti-CD4 antibody was administered in vivo at the dose of 100 μg/day three times/wk. Administration of the antibody for 1 wk resulted in almost complete elimination of CD8⁺ or CD4⁺ T cells as revealed by flow microfluorometric study (24).

Results

Mediation of Class I MHC-disparate Graft Rejection by CD8⁺ CTL-involved and CD8⁺ CTL-independent Pathways. Semipurified anti-CD8 antibody was administered into B6 recipient mice three times/wk at the dose of 100 μg/d to eliminate CD8⁺ T cells. Intraperitoneal injection of anti-CD8 antibody for 1 wk (three treatments) resulted in almost complete elimination of CD8⁺ T cells without affecting the CD4⁺ T cell subset (Fig. 1). When this procedure was continued, the CD8⁺ T cell-depleted state was maintained in the recipient B6 mice until the antibody administration was terminated (data not shown). B6 or B10.BR recipient mice depleted of CD8⁺ T cells were engrafted with skin grafts from bm1 and B10.QBR (for B6) or from B10.A and B10.AKM (for B10.BR) mice 1 wk after the initiation of anti-CD8 injection. The skin graft survival in these four sets of class I MHC-disparate combinations is shown in Fig. 2. In contrast to strikingly prolonged survival of bm1 grafts in anti-CD8 antibody-treated B6 mice (Fig. 2 *A*), grafts in the other three combinations were rejected in the antibody-treated recipients with (Fig. 2 *B*) or without only marginal prolongation of survival (Fig. 2, *C* and *D*) when compared to those engrafted in untreated recipient mice.

We examined the capacity of anti-CD8 antibody-treated recipients to generate CTL responses as well as MLR/IL-2 production against allo-class I MHC antigens. Spleen cells or lymph node cells from B6 or B10.BR recipient mice receiving the anti-CD8 injection were used as responding cells for CTL induction or MLR/IL-2 production, respectively. The results of Fig. 3 illustrate that the administration of anti-CD8 antibody results in almost complete abrogation of capacities to generate anti-allo-class I MHC CTL responses in

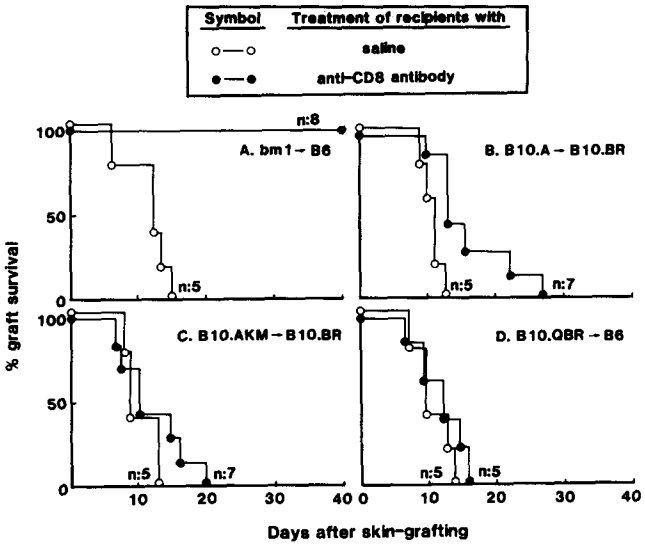

Figure 2. Effect of CD8⁺ T cell depletion on graft survival in four combinations with class I MHC disparity. Untreated or anti-CD8-treated B6 or B10.BR recipient mice were engrafted with skin grafts from indicated strains of mice 1 wk after the initiation of the anti-CD8 treatment. The antibody injection was continued after the skin grafting until the grafts were rejected.

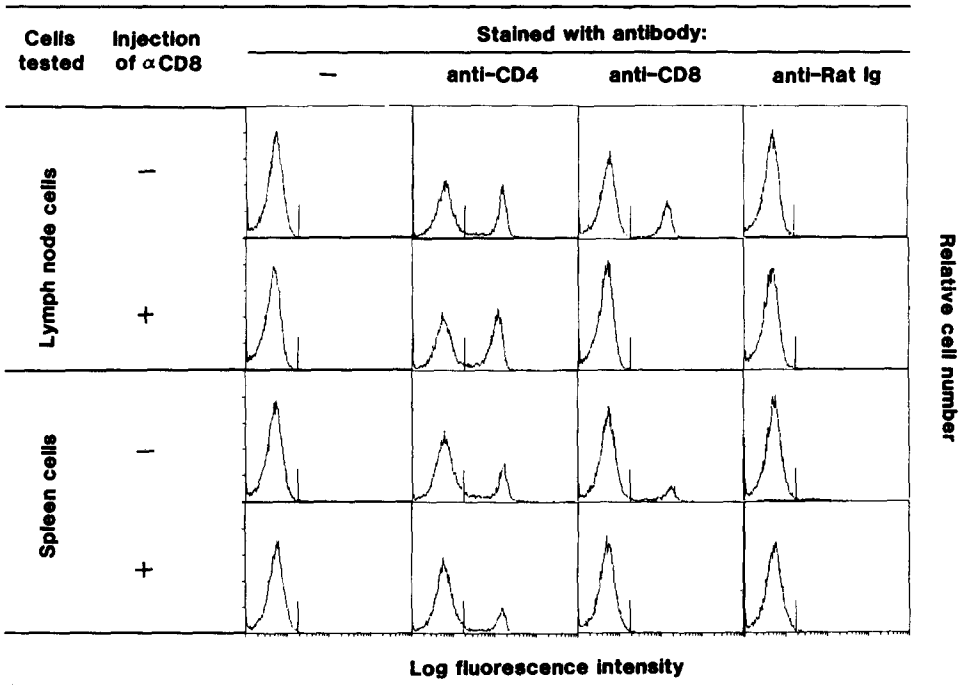

Figure 1. Depletion of CD8⁺ T cells by in vivo administration of anti-CD8 antibody. Semi-purified anti-CD8 (2.43) antibody was injected intraperitoneally at the dose of 100 μg/day three times/wk for 1 wk. Lymph node and spleen cells from these antibody-treated mice were submitted to FMF analyses.

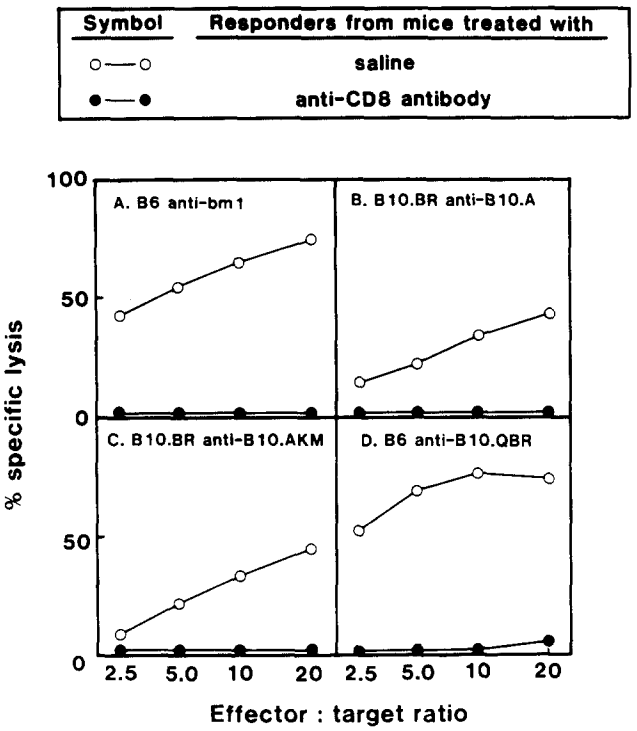

Figure 3. Effect of CD8+ T cell depletion on the generation of CTL responses in four combinations with class I MHC disparity. Spleen cells (5×10^6) from untreated B6 or B10.BR mice or mice treated with anti-CD8 antibody for 3 wk were cultured with irradiated stimulator cells (10^6) from indicated strains of mice for 5 d. Effector cells generated were assayed on Con A–induced splenic blast target cells prepared from the same strains of mice used for the in vitro stimulation.

all four sets of class I MHC-disparate combinations. In contrast, reduced but still appreciable magnitudes of MLR/IL-2 production were generated by lymphoid cells from the same CD8+ T cell–depleted mice, except in the B6-bm1 combination in which MLR and IL-2 production are mediated predominantly by CD8+ T cells (Fig. 4). It was also shown that MLRs generated in CD8+ T cell–depleted recipients are mediated by CD4+ T cells (Table 2). These results suggest that the graft rejection in class I MHC-disparate combinations does not necessarily require the operation of a classical CD8+ CTL pathway, but is also inducible by the CD8+ CTL-independent pathway that is detected as CD4+ T cell–mediated MLR/IL-2 production.

Prolonged Survival of Class I MHC-disparate Grafts by Simultaneous Suppression of Both CD8+ CTL-involved and -independent Pathways. An earlier study from our laboratory has revealed that intravenous presensitization of B6 mice with class I MHC-disparate bm1 cells induces the suppression of anti-bm1 graft rejection responses, whereas such DSP in conventional class I MHC-disparate combinations fails to reduce the graft rejection potential (21). This failure to prolong graft survival occurred despite the suppression of anti–class I MHC MLR/IL-2 production, under conditions in which an intact CTL response remained (21). In a representative conventional class I MHC-disparate combination (B6-B10.QBR), we have confirmed the effects of DSP on these immune responses. B6 mice were presensitized intravenously with 10^7 B10.QBR spleen cells, and 1 wk later, lymphoid cells were tested for their capacity to generate anti-B10.QBR MLR/IL-2 production as well as CTL responses. The results of Table 3 (Exp. 1, Group 3) and Fig. 5 (Exp. 1, Group 3) illustrate that anti-B10.QBR MLR/IL-2 production is suppressed by intravenous presensitization, whereas this regimen does not affect the induction of CTL responses. These results (Group 2) also confirm the results obtained in Figs. 3 and 4 for the effects of anti-CD8 administration on anti-B10.QBR immune responses. Almost complete inhibition of these immune responses was induced by the combination of DSP and anti-CD8 (Table 3, Group 4, Exp. 1; and Fig. 5, Group 4, Exp. 1).

Mice from the same groups as used in Table 3 and Fig. 5 were engrafted with B10.QBR or B6-C-H-2^{bm12} (bm12)

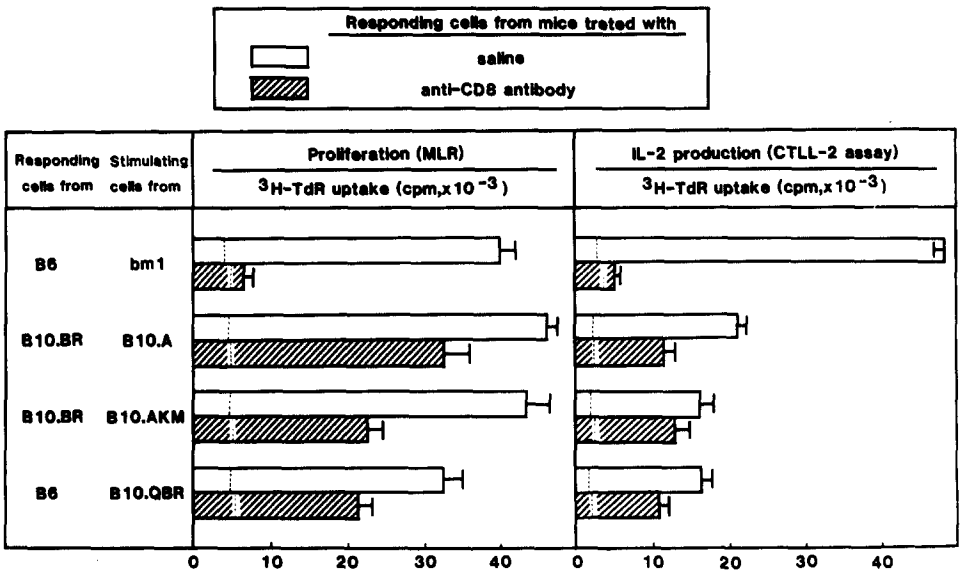

Figure 4. Effect of CD8+ T cell depletion on MLR/IL-2 production in four combinations with class I MHC disparity. Responding lymph node cells (2×10^5) from the same untreated (□) or anti-CD8-treated (▨) mice as used in Fig. 3 were stimulated with 2,000 rad X-irradiated spleen cells (4×10^5) from indicated strains of mice for 4 d. Vertical dotted lines inside each column indicate [3H]TdR uptakes in the presence of syngeneic stimulator cells as background instead of allogeneic stimulator cells.

Table 2. *CD4⁺ T Cell Mediation of MLR Generated in CD8⁺ T Cell-depleted Recipients*

Responding cells from:‡	Stimulating cells from:‡	[³H]TdR Uptake in the presence of Ab*				
		Bkg§	–	Anti-CD4	Anti-CD8	Mixture
				cpm × 10⁻³		
B6	bm1	3.8 ± 0.3	6.7 ± 0.7	2.3 ± 0.2	4.7 ± 0.5	1.1 ± 0.3
B10.BR	B10.A	5.2 ± 0.3	33.6 ± 3.4	6.1 ± 0.6	27.8 ± 5.4	4.3 ± 0.7
B10.BR	B10.AKM	5.2 ± 0.2	22.5 ± 2.4	3.4 ± 0.2	22.3 ± 3.0	3.4 ± 0.7
B6	B10.QBR	5.3 ± 0.2	20.6 ± 1.9	8.4 ± 0.3	22.7 ± 2.3	9.3 ± 0.7

* MLRs were performed in the absence or presence of anti-CD4, anti-CD8, or the mixture of these. Each antibody concentration was 100 ng/ml.
‡ Semi-purified anti-CD8 (2.43) antibody was injected intraperitoneally at the dose of 100 μg/d three times per week for 3 w. Lymph node cells (2×10^5) from those anti-CD8-treated mice were stimulated in vitro with 2,000 rad X-irradiated indicated strains of spleen cells (4×10^5) for 4 d.
§ Responding cells were cultured with syngeneic spleen cells as background (Bkg).

control skin grafts 1 wk after DSP with B10.QBR cells. After B10.QBR skin grafting, the administration of anti-CD8 antibody was continued in Groups 2 and 4. Prolongation of B10.QBR skin grafts was produced in the group receiving the combined treatment (Group 4) (Fig. 6 *A*). Such prolongation was found to be specific for B10.QBR used for DSP, since the same combined treatment did not affect the rejection of control bm12 grafts (Fig. 6 *B*).

Additional experiments were performed to examine the capacities of four groups of mice to generate MLR/IL-2 production and CTL responses 1 wk after B10.QBR skin grafting. The results of Table 3, Exp. 2; and Fig. 5, Exp. 2 illustrate that these immune responses against B10.QBR are still suppressed in B10.QBR skin-bearing B6 mice receiving the combined treatment (Group 4). Thus, taken collectively, the results of Table 3 and Figs. 5 and 6 indicate that DSP is capable of suppressing CD8⁺ CTL-uninvolved graft rejection pathway(s) and that the inhibition of the overall class I–disparate graft rejection potential is inducible when the regimen for eliminating a CD8⁺ CTL-involved graft rejection element is combined with DSP capable of abrogating CD8⁺ CTL-independent pathway(s).

Prolongation of Class I MHC-disparate Graft Survival by the Combination of DSP with anti-CD4 Antibody Injection. We next investigated whether the CD8⁺ CTL-involved pathway leading to graft rejection is abrogated by elimination of CD4⁺ T cells. Anti-CD4 antibody was administered to B6 mice in a protocol similar to that used for anti-CD8 antibody. 1 wk after initiation of anti-CD4 treatment, DSP with B10.QBR cells was performed. Another 1 wk after the DSP,

Table 3. *Effect of Anti-CD8 Treatment and/or DSP on MLR in a Class I MHC-disparate Combination*

Exp. (stage‡)	Group	Responding cells from B6*			MLR		IL-2 production	
		α-CD8 Ab	DSP	Grafting	B6	B10.QBR	B6	B10.QBR
						cpm × 10⁻³		
1	1	–	–	–	2.9 ± 0.5	17.9 ± 2.7	0.2 ± 0.1	9.9 ± 0.1
	2	+	–	–	2.1 ± 0.3	9.3 ± 0.5	0.2 ± 0.1	4.5 ± 0.1
	3	–	+	–	2.4 ± 0.6	3.0 ± 0.7	0.2 ± 0.1	1.8 ± 0.1
	4	+	+	–	2.7 ± 1.0	3.9 ± 0.4	0.2 ± 0.0	1.1 ± 0.1
2	1	–	–	+	2.0 ± 0.4	23.3 ± 3.5	0.2 ± 0.0	23.1 ± 0.6
	2	+	–	+	1.6 ± 0.1	11.4 ± 1.4	0.3 ± 0.0	11.2 ± 1.0
	3	–	+	+	1.6 ± 0.6	3.3 ± 0.7	0.2 ± 0.0	1.5 ± 0.0
	4	+	+	+	2.0 ± 0.3	3.2 ± 1.0	0.3 ± 0.1	1.0 ± 0.1

* Recipient B6 mice received the DSP with B10.QBR cells 1 wk after the initiation of anti-CD8 antibody administration. Half of mice in each group were submitted to MLR and IL-2 production after the DSP (Exp. 1), and the other half of mice were engrafted with B10.QBR skin grafts 1 wk after the DSP. An additional 1 wk later, these mice were used as the source of responding cells in Exp. 2.
‡ Responding lymph node cells were obtained 1 wk after DSP (Exp. 1) or skin grafting (Exp. 2).

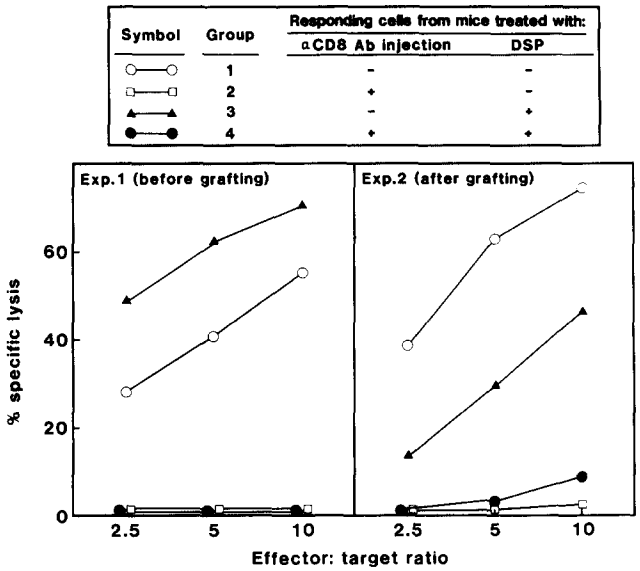

Figure 5. Effect of anti-CD8 treatment and/or DSP on CTL induction in a class I MHC-disparate combination. Spleen cells (5×10^6) from the same B6 mice as used in Exps. 1 and 2 of Table 3 were used as a source of responding cells for CTL induction and stimulated with B10.QBR cells (4×10^5).

these mice were engrafted with B10.QBR grafts. The administration of anti-CD4 or anti-CD8 antibody was continued three times/wk at the dose of 100 μg/d (Fig. 7). The combination of DSP with anti-CD8 injection again produced prolongation of B10.QBR graft survival. The administration of anti-CD4 antibody also induced an appreciable prolongation of B10.QBR graft survival, although this effect was weaker than that observed for the combination of DSP and anti-CD8 treatment.

Time-related Difference between the Effect of CD8⁺ and CD4⁺ T Cell Depletion in Combination with DSP. We finally asked whether there are differential requirements for the effect of CD8⁺ vs. CD4⁺ T cell depletion in combination with DSP for reducing CTL and graft rejection responses. B6 mice were engrafted with B10.QBR grafts 1 wk after the B10.QBR DSP, and treatment with anti-CD4 or anti-CD8 antibody was initiated simultaneously with grafting. As shown in Fig. 8, administration of anti-CD8 by this schedule produced prolongation of B10.QBR graft survival comparable to that observed when anti-CD8 treatment was begun 2 wk before grafting (Figs. 6 *A* and 7). In contrast, the administration of anti-CD4 antibody initiated at the timing of grafting produced no prolonged graft survival.

To investigate the cellular mechanisms underlying the effect of treatment schedule on the consequences of anti-CD4 antibody treatments, we examined the CTL responses of two groups of mice in which anti-CD4 antibody administration was started either before or after DSP. Spleen cells were obtained 2 wk after DSP and tested for the presence of CD4⁺ T cells (Fig. 9) and for the capacity to generate anti-B10.QBR CTL responses (Fig. 10). These results demonstrate that: (*a*) depletion of CD4⁺ T cells is equally effective irrespective of whether anti-CD4 treatment is initiated after (Group 4) or before DSP (Group 5) (Fig. 9); and (*b*) elimination of CTL responses by treatment with anti-CD4 antibody depends on initiation of antibody administration before DSP. This suggests that under conditions in which CD8⁺ Th are eliminated by DSP, anti-CD4 antibody interferes with the activation of CD8⁺ CTL precursors by eliminating an alternate Th cell, CD4⁺ CTL helpers.

Discussion

A major issue in transplantation immunology is to analyze the cellular interactions underlying in vivo graft rejection responses. Allograft rejection results from interactions between functionally and phenotypically distinct subsets of alloantigen-reactive T cells (4). Even in donor-host combinations with only class I MHC disparity, the exact cellular mechanisms remain to be clarified (vide infra).

Another important practical goal is establishment of manipulations by which donor-specific alloreactivity can be abrogated. Attempts have been made to induce alloantigen-specific unresponsiveness by introduction of alloantigens via an intravenous route, as exemplified by the beneficial effect of pretransplant DST in human studies (5–10). However, in some cases, DST fails to reduce the recipient's alloreactivity but rather sensitizes the host to the relevant alloantigens (11–15). Different outcomes of attempts at immune modulation of host reactivity have also been observed in animal studies using DSP as a model of DST (16–21). Thus, it remains im-

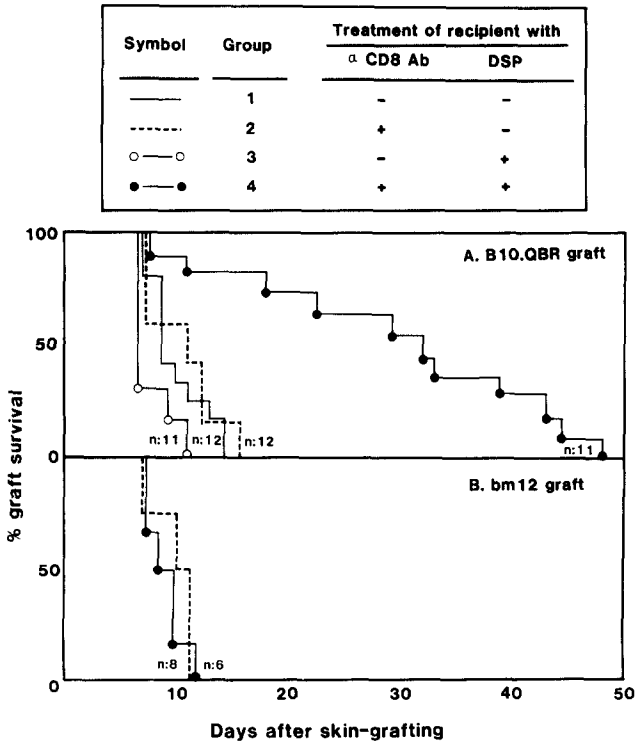

Figure 6. Effect of anti-CD8 treatment and/or DSP on the generation of graft rejection responses. B6 mice received either anti-CD8 treatment, DSP with B10.QBR cells, or the combination of these. 1 wk after the DSP, mice were engrafted with B10.QBR or bm12 (control) skin grafts.

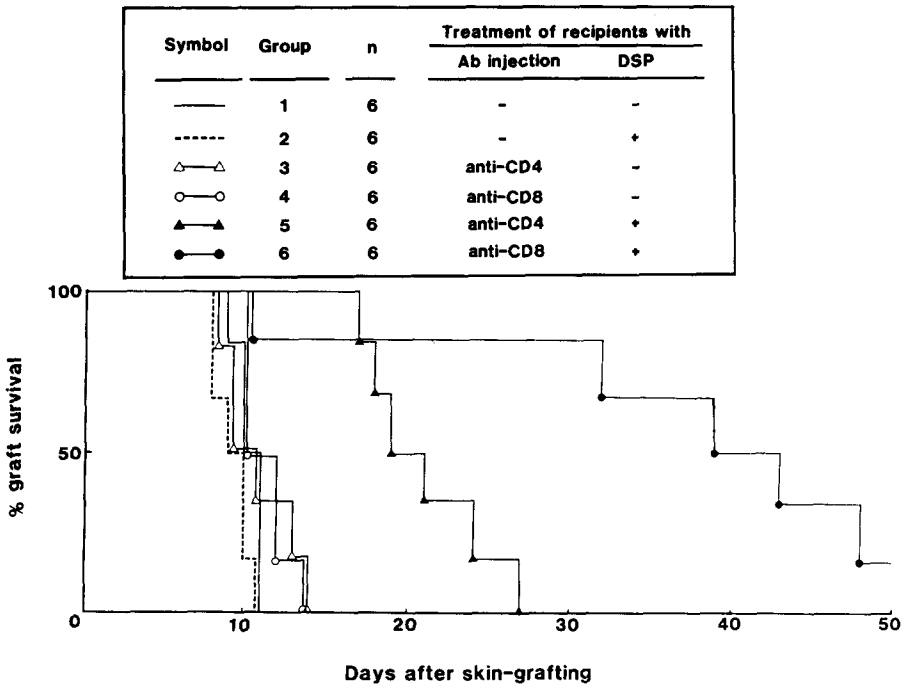

Figure 7. Effect of anti-CD4/anti-CD8 treatment and/or DSP on the generation of graft rejection responses. The protocol was essentially the same as used in Table 3 and Fig. 6, except for continued injection of the same dose of anti-CD4 antibody instead of anti-CD8 antibody in some groups.

portant to identify DSP/DST-resistant elements of anti-allo responses and to develop means to reduce these elements of alloreactivity.

In the present study, we investigated the heterogeneity of the cellular mechanisms of graft rejection responses in class I MHC-disparate combinations and the effects of DSP on each element of anti-class I MHC immune responses. The results demonstrate that: (*a*) graft rejection responses in conventional class I MHC disparities consist of classical $CD8^+$ CTL-involved as well as CTL-independent pathways; (*b*) these $CD8^+$ CTL-involved and -independent pathways are, respectively, resistant and susceptible to DSP; (*c*) the overall suppression of graft rejection responses is inducible by the combination of DSP with elimination of the $CD8^+$ CTL-involved pathway; and (*d*) the reduction of $CD8^+$ CTL responses is achieved by eliminating either $CD8^+$ CTL pre-

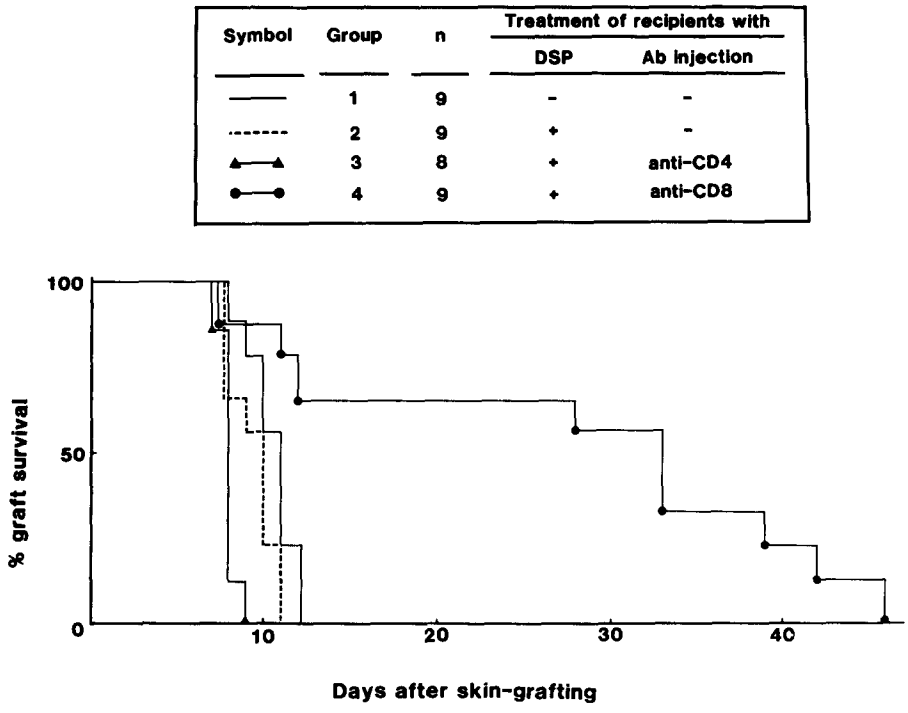

Figure 8. Anti-CD4 treatment initiated after DSP fails to induce the prolongation of graft survival. B6 mice were engrafted with B10.QBR skin grafts 1 wk after the DSP with B10.QBR cells. Simultaneously with skin grafting, the administration of anti-CD4 or anti-CD8 antibody was started.

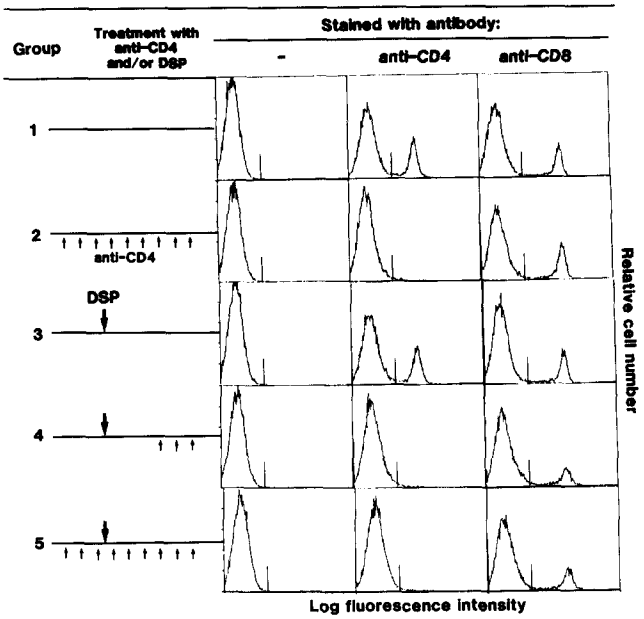

Figure 9. Depletion of CD4+ T cells by in vivo administration of anti-CD4 antibody. B6 mice were untreated (Group 1), received anti-CD4 antibody 9 times for 3 wk (Group 2), received DSP alone (Group 3), received DSP and anti-CD4 injection after DSP (three times for the final 1 wk) (Group 4), or received DSP and anti-CD4 antibody injection nine times for 3 wk (Group 5). Lymph node and spleen cells from these five groups of mice were submitted to FMF analyses.

cursors/effectors or CD4+ CTL helpers. Thus, the present study indicates the operation of heterogeneous graft rejection pathways in responses to class I MHC disparity and the differential susceptibility of these pathways to immunomodulation induced by DSP/DST.

It would be of value to summarize the cellular elements involved in the complicated network of graft rejection responses. While CD8+ CTL have been generally regarded as representing the major effector mechanism (26, 27), recent studies have revealed the existence of various subsets of Th cells that function to activate CD8+ CTL precursors (1–3). These include allo class I MHC-restricted CD8+ Th cells; allo class I MHC-reactive self Ia-restricted CD4+ Th cells; and allo class II MHC-restricted CD4+ Th cells. In combinations with only class I MHC disparity, the former two Th cell subsets are activated to recognize allo class I MHC antigens, although predominatly allo class I MHC-restricted CD8+ Th cells function in the B6-bm1 combination where class I disparity is generated by a simple mutation in a class I MHC gene (16, 18). In addition to this classical CD8+ CTL-involved pathway, it is likely that tissue destruction/graft rejection in transplantation immunity, as well as tumor immunity and autoimmunity, is also induced by an alternate immune pathway(s) (28–36). The cellular and molecular mechanisms involved in such pathway(s) remain to be determined. However, potent rejection of class I–disparate grafts in CD8+ T cell–depleted recipients confirms the operation of a CD8+ CTL-independent pathway that is mediated by the CD4+ T cell subset.

It appears that DSP induces elimination or functional inactivation of particular T cell subsets. Earlier studies from our laboratory have demonstrated that DSP results in almost complete inactivation of CD8+ Th cells but not of CD8+ CTL precursors (18, 37); i.e., although the frequency of CD8+ CTL precursors is appreciably reduced by a single DSP (18), all precursors are not eliminated and some of these remaining after DSP are activated to induce graft rejection when an alternate Th cell pathway is introduced (37). DSP also affects some CD4+ T cells. As shown in our previous work (21) and this study, CD4+ as well as CD8+ T cell subpopulations as detected by MLR/IL-2 production are susceptible to the effects of DSP. Of these DSP-susceptible subpopulations, at least the CD4+ T cell subpopulation is capable of inducing graft rejection. This was demonstrated by the finding presented here that the capacity of CD8+ T cell–depleted mice to produce graft rejection was abrogated by DSP. In contrast, a CD4+ T cell subpopulation whose activity is monitored by the ability to assist the activation of CTL precursors is resistant to the DSP effect. This was also shown in the present study by demonstrating that DSP

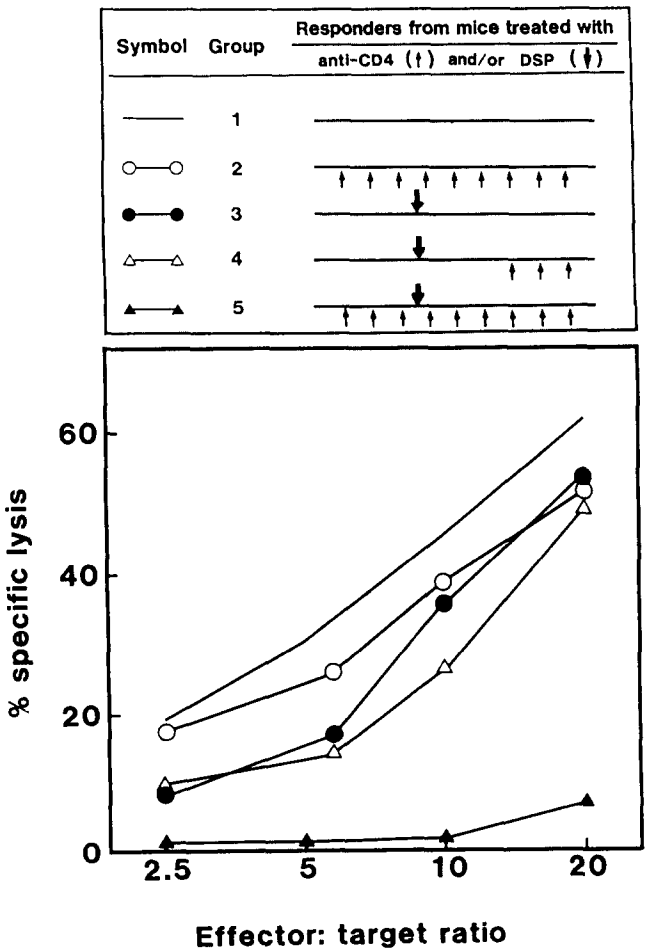

Figure 10. Requirement of pre-DSP anti-CD4 treatment of reducing the generation of CTL responses. Spleen cells (5×10^6) from the same five groups of mice as used in Fig. 9 were stimulated with B10.QBR cells (10^6).

Table 4. *Phenotypically and Functionally Distinct T Cell Subsets Activated by Class I MHC Alloantigens and Involved in Class I MHC-disparate Skin Graft Rejection*

Phenotype	Function	Capacity to exhibit MLR	Resistance to DSP	Participation in class I MHC-disparate allograft rejection	
				CTL-involved	CTL-independent
CD8	CTL-p	−	+	+	−
CD8	CTL-Th	+	−	+	−
CD4	CTL-Th	−	+	+	−
CD4	Effector/inducer	+	−	−	+

capable of abrogating the MLR/IL-2-producing capacities of the above $CD4^+$ and $CD8^+$ T cell subpopulations, including allo class I MHC-restricted $CD8^+$ Th cells, failed to eliminate CTL as well as graft rejection responses. Thus, the present study using two immunomodulating regimens (anti-CD8 antibody treatment and DSP) may divide graft rejection-contributing T cells into four functionally and phenotypically distinct subsets (See Table 4); (*a*) $CD8^+$ CTL precursors, (*b*) $CD8^+$ allo class I-restricted Th cells with MLR/IL-2-producing capacities, (*c*) $CD4^+$ T cells that are not detected by MLR/IL-2 production but represent allo class I-reactive self Ia-restricted CTL-Th cells; and (*d*) $CD4^+$ T cells with MLR/IL-2-producing capacities.

The present study indicates that while two of the above-mentioned allo class I-reactive T cells (*b* and *d*) are susceptible to DSP, the rest of the elements participating in CTL responses are resistant to DSP. Therefore, it is reasonable to assume that in class I MHC-disparate combinations, especially in which potent CTL responses are induced, DSP is not sufficient alone to prolong graft survival, but is effective in reducing graft rejection responses in vivo only when combined with treatments eliminating either $CD8^+$ CTL precursors or $CD4^+$ CTL-Th cells. It should be noted that injection of anti-CD8 antibody either at the time of DSP or at the time of grafting was effective in reducing CTL responses, whereas administration of anti-CD4 antibody was effective for reducing the generation of CTL responses only when it was started before DSP. This is compatible with the notion that the anti-CD4 antibody treatment exerts its suppressive effects on the activation of $CD8^+$ CTL precursors by eliminating previously DSP-resistant $CD4^+$ CTL helpers (allo class I MHC-reactive self Ia-restricted). The fact that the administration of anti-CD4 antibody after DSP is not effective may suggest that DSP capable of inducing concomitant inactivation of $CD8^+$ Th cells sensitizes $CD8^+$ CTL precursors unless allo class I-reactive self Ia-restricted $CD4^+$ Th cells are inactivated beforehand, and that $CD8^+$ CTL, once activated, function in a manner independent of $CD4^+$ Th cells (Table 4).

The present observation that the prolongation of graft survival is induced by the combination of DSP and elimination of DSP-resistant elements may be discussed from the following two perspectives. First, although such combined treatment induced the considerable or appreciable prolongation of graft survival, most grafts were rejected 3–6 wk after grafting. The limited term of the prolongation was also observed in our initial studies using the B6-bm1 and B6-bm12 combinations. Our study revealed that the survival of bm1 or bm12 grafts in the DSP model was strikingly prolonged by pre-DSP thymectomy, indicating that anti-allo tolerance once induced by DSP is broken down by the repopulation of new clones from the thymus (38). Thus, the limitation of graft survival period prolongation in the present models may be explained by the recovery of $CD8^+$ or $CD4^+$ T cells from the thymus under conditions in which the respective anti-CD4 or anti-CD8 treatment is continued. Differences in the contribution to the prolongation of graft survival between $CD4^+$ and $CD8^+$ T cell depletion combined with DSP could also be accounted for by postulating differential requirements of the thymus for repopulating T cells with different phenotypes and functions (38–42).

Another important aspect concerns potential clinical application of attempts to inhibit some elements of DSP-resistant CTL responses in individuals receiving DSP. In considering the difficulty in continuing the injection of antibody in humans, utilization of substitutes for antibodies could be a more practical approach for improving the DST effect. In this context, it would be of great value to mention our studies that are currently being performed (Iwata, H., S. Kitagawa, S. Sato, H. Hirose, T. Hamaoka, G.M. Shearer, and H. Fujiwara, manuscript in preparation; and Iwata, H., S. Kitagawa, S. Sato, H. Hirose, T. Hamaoka, and H. Fujiwara, manuscript in preparation). Cyclosporin A (CsA) is a powerful immunosuppressive drug that has been widely used in organ transplantation in the past decade (43). This drug and a recently developed drug, FK506 (44, 45), have similar functional properties and alleviate allograft rejection by inhibiting the activation of Th cells (43, 44). Which of the three above-mentioned allo-reactive Th cell subsets is the most susceptible to the inhibiting effect of these drugs was examined. We have found that the activity of allo class I MHC-reactive self Ia-restricted $CD4^+$ CTL-Th cells was the most sensitive to FK506 and that FK506 in suboptimal doses selectively abrogated the function of this Th cell subset (Iwata, H., S. Kitagawa, S. Sato, H. Hirose, T. Hamaoka, G.M. Shearer, and H. Fujiwara, manuscript in preparation). Our study fur-

ther revealed that the combination of DSP with the utilization of suboptimal doses of FK506 produced striking prolongation of class I–disparate graft survival under conditions in which either of these two regimens alone exhibited only a marginal effect (Iwata, H., S. Kitagawa, S. Sato, H. Hirose, T. Hamaoka, and H. Fujiwara, manuscript in preparation). Thus, these observations may provide an important implication for developing realistic approaches to regulate graft rejection responses by using suboptimal doses of immunosuppressive drugs.

Our present results illustrate that heterogenous graft rejection pathways function in class I MHC-disparate donor-host combinations and that DSP as a model of DST is effective for modulating one of these pathways. The results also show that a classical $CD8^+$ CTL-involved pathway is resistant to DSP and that DSP has a beneficial effect only when immunomodulation of the DSP-resistant elements is achieved. Thus, by demonstrating the existence of alternative pathways for graft rejection, and by suggesting approaches for immunomodulation of the DSP-resistant pathway, the present study could provide an important insight into future transplantation immunology.

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