INDUCTION OF CLASSICAL TRANSPLANTATION TOLERANCE IN THE ADULT

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Clinical organ transplantation between genetically disparate individuals presently requires nonspecific immunosuppressive agents to prevent rejection. However, the use of such agents risks morbidity and mortality from a range of associated side effects and does not always guarantee success of the graft. Therefore, there is a need to establish methods of "donor-specific transplantation tolerance" that would not require long-term immunosuppressive therapy. Since the first report of specific transplantation tolerance, induced in newborn mice by injection of donor bone marrow (BM)[1](1), there has been much interest in achieving the same in the adult. If it were possible to recreate in the adult the immunological naivety of the neonate, then similar marrow transplants could be used to tolerize for organ grafts.

Many attempts have been made to do this. At the experimental level the most successful have involved the use of lethal whole body irradiation (2, 3); total lymphoid irradiation (4); ablative chemotherapy (5); antilymphocyte antisera (6, 7) and in one case of P1→(P1 × P2)F_1 combination, by using antidonor MHC mAbs (8); all coupled with transplantation of BM from the allogeneic donor. In essence, the common theme of these strategies was to allow the donor marrow to establish some degree of hemopoietic chimerism. The ubiquitous distribution of chimeric hemopoietic cells clearly favors them as vehicles to present donor antigens for tolerance whatever the mechanisms involved.

We demonstrate here that transplantation tolerance can, in a number of strain combinations, be induced in the adult mouse by combining BM transplantation (BMT) together with parenteral administration of CD4 and CD8 mAbs. We show this for combinations differing in multiple "minor" transplantation antigens and for combinations differing in minors plus class I MHC. We investigate the need for donor and recipient T lymphocyte depletion; document true tolerance in recipient T cells; and provide evidence suggesting differential handling of minor vs. major histocompatibility antigens in the antibody-treated recipients. All this was possible in animals that had not received either irradiation or myelosuppressive drugs. Surprisingly, T cell tolerance across Mls differences was not accompanied by any reduc-

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[1] *Abbreviations used in this paper:* BM, bone marrow; BMT, bone marrow transplantation; MST, median survival time; rIg, rat immunoglobulin.

tion in expression of Vβ6 gene products in the peripheral immune system. These findings should serve as a basis for the evolution of improved strategies to impose tolerance for successful organ transplantation.

Materials and Methods

Animals. Mouse strains used in the experiments are listed in Table I. They were bred and kept in the conventional facilities of the Pathology Department, Cambridge University, and were used in sex- and age-matched groups.

Monoclonal Antibodies. The two pairs of synergistic mAbs used in the experiments were all of rat IgG (rIgG)2b isotype. YTS 191.1 and YTA 3.1 are CD4 specific (references 9, 10). Of the two CD8 mAbs, YTS 169.4 is anti-Lyt-2 and YTS 156.7 is anti-Lyt3. (reference 9 and unpublished data). The rIgG2a mAbs YTS 177.9 (CD4) and YTS 105.18 (anti-Lyt-2) known to be nondepleting antibodies were developed in this laboratory (Qin, S., S. Cobbold, Y. M. Kong, H. Waldmann). All antibodies were obtained from (DA × LOU)F_1 rat ascitic fluid, purified by ammonium sulfate precipitation and dialyzed into PBS.

Preparation of mAb F(ab')$_2$ Fragments. F(ab')$_2$ fragments of YTS 191.1 and YTS 169.4 were made by the method of Rousseaux et al. (11). Antibodies in 0.1M sodium phosphate were digested with Type XVII protease from *Staphylococcus aureus* (Strain V8; Sigma Chemical Co., Poole, UK) at an enzyme/substrate ratio of 1:200 (wt/wt). The digestion was carried out at 37°C for 4 h, stopped by freezing. Antibody F(ab')$_2$ fragments were purified by gel filtration on a 2.5 × 95 cm column packed with Ultragel AcA 44 (LKB, Villeneuve-la-Gavenne, France). The purity of F(ab')$_2$ products was confirmed by SDS-PAGE and by complete inhibition of in vitro complement lysis by parental antibodies as reported elsewhere (12).

Bone Marrow Transplantation. Bone marrow cells were flushed from donor femoral and tibial bones and washed with cold Eagles' Hepes medium. Live cells were counted with trypan blue, and adjusted to appropriate viable cell concentrations. T cell-depleted BM were obtained from thymectomized mice that had been given two CD4 and CD8 synergistic mAb pairs (2 mg/mouse, i.e., 0.5 mg of each mAb), or from normal mice given the mAbs 2 d before they were killed. In some experiments, an equal number of spleen cells from the same marrow donors were added into the inoculum. 0.2 ml of the cell suspensions was injected intravenously into recipient mice.

Skin Grafting. Skin grafts were performed as previously reported. (9) In short, donor tail skin grafts (0.5 cm × 0.5 cm) were transplanted onto the lateral thoracic wall of the recipient, then covered with clean gauze and plaster for 7 d. The graft survival was documented daily thereafter, and the results were analyzed by Log-Rank method (13).

Mixed Lymphocyte Culture and Cell-mediated Cytotoxicity In Vitro. To achieve an adequate in vitro response to minor histocompatibility antigens, responder mice were primed intraperitoneally with irradiated spleen cells of the stimulator strain 1 wk before testing. In MLC, responder spleen cells (2×10^6/ml) were incubated with irradiated (3,000 rad) stimulator cells in Iscove's modified Dulbecco's medium containing 5% FCS at 37°C, 5% CO_2 in a humidified incubator. On day 3, 5 μl ^{125}I-deoxyuridine (10 μCi/ml; Amersham Corp.,

TABLE I

Mouse Strains Used in the Experiments

	H-2	
	K A E D	"Background"
CBA/Ca	k k k k	CBA, Mls^b
B10.BR	k k k k	B10
B10.A	k k k d	B10
B10.D2	d d d d	B10
BALB/c	d d d d	BALB
BALB/k	k k k k	BALB
AKR/J	k k k k	Mls^a

Amersham, UK) was added into each well. The cells were harvested 6 h later, ^{125}I incorporation was measured on a Philips Automatic Gamma Counter (Philips, Eindhoven, The Netherlands). The cytotoxic responses were generated by incubating the responder cells with stimulator cells as for the MLC, but in RPMI 1640, 5% FCS for 6 d in 24-well Linbro plates. Cells were harvested, washed twice, and resuspended in U-bottomed 96-well microtiter plates. Con A blast target cells (10^6/ml) labeled with 30 μCi/ml ^{51}Cr (Amersham Corp.) were incubated with various number of effector cells (final volume, 200 μl/well) for 4 h. 100 μl of supernatant was then collected and the radioactivity was counted on a Philips Automatic Gamma Counter. The specific release was calculated by the following formula: Percent specific release = $100 \times [(e\text{-}s)/(t\text{-}s)]$. where e is the sample count per minute, s is the spontaneous release of target cells in medium only, t is the total counts by resuspending cells after incubation. All samples were performed in triplicates.

Detection of Donor Chimerism. The demonstration of donor B cell chimerism was based on a difference in Igh1 loci (encoding IgG2a) between CBA (Igh1a) and B10.BR (Igh1b), by a hemagglutination inhibition assay. Normal human RBC washed in saline were coupled with a mouse IgG2a mAb (MAR 18.5, Igh1b, reference 14) in the presence of chromic chloride. Sample sera were diluted in flexible U-bottomed plates (Falcon Labware, Oxnard, CA) with a rabbit anti-mouse Igh1b antiserum (a kind gift from Dr. C. Elson, Bristol University, Bristol, UK). After 10 min of incubation, Igh1b RBC were added into each well, and the agglutination results were observed 1 h later. A series of CBA sera laced with a defined amount of B10.BR serum were used in each assay to give a standard inhibition curve from which the percentage of donor Ig chimerism was calculated. This method enabled us to detect down to 0.2% B10.BR serum.

To follow T cell chimerism, AKR/J BM cells were injected into CBA/Ca mice along with the mAb treatment regime. 4 wk after transplant, the mice were bled and peripheral mononucleated cells were stained with biotinylated anti-Thy-1.1 and anti-Thy-1.2 (reference 15) mAbs, followed by streptavidin-FITC (Amersham Corp.). The percentage of binding was analyzed by a Cytofluorograf (model 50-H; Ortho Diagnostic Systems, Inc., Westwood, MA) and Ortho 1250 computer.

Two-Color Flow Cytometry. Mouse lymph node cells were incubated with CD4 (YTS 191.1 + YTA 3.1) or CD8 (YTS 169.4 + YTS 156.7) in spent tissue culture medium. Biotinylated 44-22-1 (16) was used to stain Vβ6 chain of the T cell receptor. The green fluorescence was developed by FITC-conjugated Norig 7.16 (anti-rat IgG2b, reference 17), the red fluorescence by streptavidin-phycoerythrin (Serotec, Kidlington, UK). The data were analyzed by a Cytofluorograf (Ortho Diagnostic Systems, Inc.), and 1250 computer.

Results

The Effect of CD4 and CD8 mAb Therapy on Skin Graft Rejection. The following experiments analyze the consequences of administering immunosuppressive antibodies together with transplants of donor mouse BM on survival of donor-type skin grafts. To clearly distinguish the effects of the combination (mAbs + BM) from that of the antibodies alone, we first determined how long the immunosuppressive effects of the mAb cocktail would last. This we did by grafting allogeneic skin at different times after the antibody injection. The model system used B10.BR (H-2^k) tail skin grafts onto CBA/Ca (H-2^k) recipients; in other words, incompatibility at multiple minor loci. Normal CBA/Ca mice mount a rapid rejection response to B10.BR skin with a median survival time (MST) of 10 d (Fig. 1). The mAb cocktail of two CD4 (YTS 191.1 and YTA 3.1) and two CD8 mAbs (YTS 169.4 and YTS 156.7) reactive to nonoverlapping epitopes of the CD4 and CD8 molecules were injected to achieve maximal immunosuppression. A mixture of 1.2 mg (0.3 mg of each of the four) mAb was given in three injections over 5 d. As can be seen in Fig. 1, this treatment significantly prolonged the MST of grafts transplanted up to 2 wk after antibody injection. However, first set skin grafts applied 3 wk after the completion of mAb

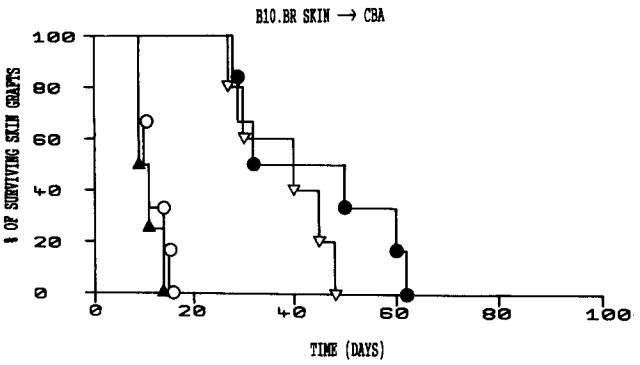

FIGURE 1. Rejection of B10.BR skin grafts by CBA/Ca mice at different times after CD4 and CD8 antibody treatment. CBA/Ca (n = 6) mice were injected with three doses of CD4 and CD8 mAbs over 5 d. B10.BR skin grafts were given at 1 wk (●), 2 wk (▽) and 3 wk (○) after the last mAb injection. The control group (▲) received no antibody. Analyses of MST: controls versus mAb + skin graft at 1 wk, $p \leq 0.007$; vs. 2-wk grafts, $p \leq 0.01$; vs. 3-wk grafts $p \leq 0.7$.

therapy were rejected as rapidly (MST, 10.5 d) as grafts onto untreated recipients. This return of immunocompetence allowed us to rapidly assess the tolerizing potential of BM when added to the same mAb cocktail. This we could do by performing skin transplantation 3 wk after administration of the inoculum. Any delay beyond the control time would then reflect the tolerizing contribution of the BM.

CD4 and CD8 mAb Therapy Permits the Development of Donor-specific Tolerance after Bone Marrow Transplantation. T cell-depleted B10.BR BM and spleen cells were infused into CBA/Ca mice under the umbrella of mAb therapy given as above. Mice so treated accepted B10.BR skin grafts applied 3 wk later (MST >200 d) (Fig. 2 *a*). Spleen cells were included in the inocula simply to maintain the comparability to the classical model. We later established that bone marrow cells alone were sufficient to induce tolerance and therefore spleen cells were omitted from most of the subsequent experiments.

All three elements of the tolerizing inoculum (the two sets of mAbs and the marrow cells) were essential. Mice given marrow and no mAb or marrow and only one of the sets of mAbs (CD4 or CD8) rejected the skin within 20 d (Fig. 2 *b*). Substitution of the marrow with another source of donor antigen (skin) was ineffective (Fig. 2 *c*). In fact, grafting the test (second) skin resulted in prompt rejection of both the first and the test grafts.

To establish whether the prolonged graft survival was truly a result of tolerance rather than of graft immunogenicity decaying with time, second B10.BR grafts were transplanted 130 d after the first. These were all accepted together with continued survival of the first grafts. Clearly, the recipient mice were tolerant of donor tissue (Fig. 2 *b*).

Further studies (our unpublished data) showed that for this strain combination at least 10^6 T-depleted marrow cells and at least an average of 250 μg of each of the mAb were necessary. We did not separately determine the relative requirements for CD4 and CD8 mAbs. However, our previous work (9) has documented that <10 μg of CD8 mAb (YTS 169.4) is an effective depleting dose; whereas at least 200 μg of the CD4 mAb (YTS 191.1) is required for equivalent depletion. Therefore, the present results probably reflect the requirement for a high CD4 mAb dose.

Thus far we have, for reasons of interpretation, purposely separated the marrow/mAb

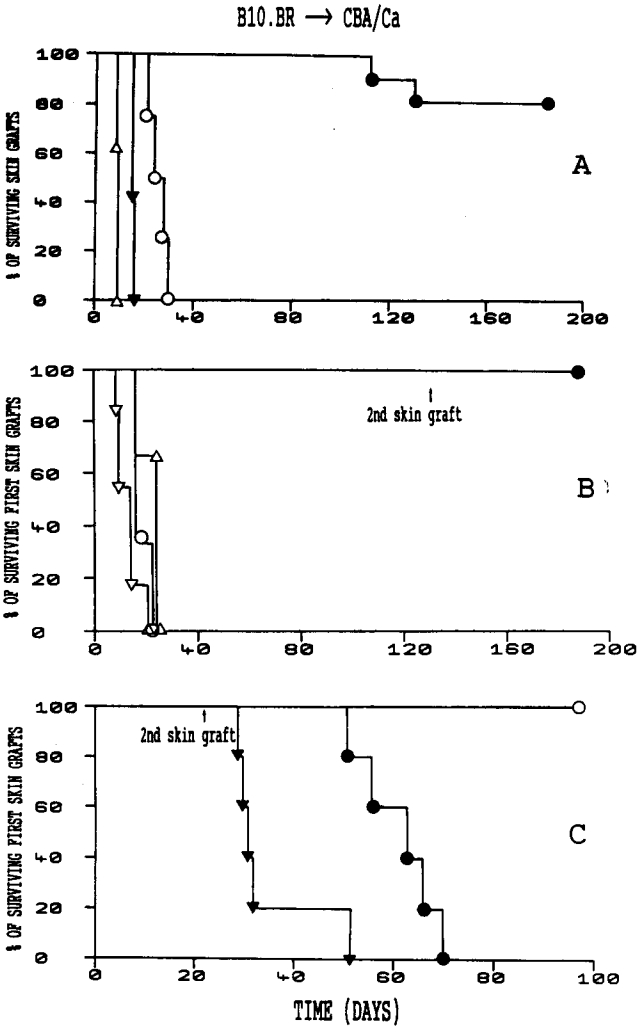

FIGURE 2. Tolerance to multiple minor transplantation antigens induced by the combination of CD4/CD8 mAb therapy and BMT. (*A*) Normal CBA/Ca (n = 12) mice were given a total of 1.2 mg of CD4 and CD8 mAbs on days 0, 3, and 5, plus 10^7 BM and 10^7 splenic B10.BR T-depleted cells 2 h after the first mAb injection. B10.BR skin was grafted 3 wk later. MST: tolerant mice (●) >300 d; control groups received antibodies only (○, n = 6) 16 d; BM and spleen cells only (△, n = 6) 8 d. All groups rejected BALB/c skin within 12 d (▼). (*B*) CBA/Ca mice (n = 6) received a total of 2.4 mg CD4 mAbs (1.2 mg of YTS 191.4 and 1.2 mg YTA 3.1; △); 2.4 mg of CD8 mAbs (1.2 mg of YTS 169.4 and 1.2 mg YTS 156.7; ○); 2.4 mg of CD4 and CD8 mAbs (0.6 mg of each of the four mAbs; ●) in three injections as above or no antibody (▽). B10.BR BM/ spleen cells and skin were given as above. MST: CD4-depleted, 16 d; CD8-depleted, 16 d. In the group maintaining long-term grafts (●), a second B10.BR skin graft was transplanted 130 d after the first. Both the first and second grafts on each animal (n = 6) have remained healthy for a further 200 d ($p \leq 0.001$). (*C*) Three doses of CD4 and CD8 mAbs were given to CBA/Ca mice as before. On the day of the first antibody injection, B10.BR skin grafts were transplanted onto two groups of mice (six mice/group). Another group of six CD4+CD8-depleted mice received instead of skin, 2×10^7 BM and spleen cells from T-depleted B10.BR mice. 3 wk later, the BMT group (○) and a skin-bearing group (▼) were grafted with B10.BR tail skin. Animals that received the second skin grafts rejected both the original and the test grafts simultaneously. MST: original grafts, 31 d; test grafts, 9 d; single skin graft group (●) 63 d ($p \leq 0.06$); mAb+BMT group ≥100 d.

induction therapy from the skin graft readout by a 3-wk interval. In practice, the whole induction protocol and test skin graft (at least for the B10.BR into CBA/Ca combination) can be compressed into a single day and tolerance can be achieved as easily (Table II). This finding has obvious clinical implications. On the other hand, the skin transplant can be postponed for up to 12 mo after BMT, with skin graft survival of over a further 100 d (Table II), suggesting the tolerizing effect of the present regime can be both immediate and long lasting.

TABLE II

Time Between BMT, mAb Therapy, and Skin Grafting for Successful Tolerance Induction

mAb on days	BMT on days	Skin grafting on days	MST
0,3,5	0	0	>300
0,3,5	0	28	>300
0,3,5	0	180	>200
0,3,5	0	370	>100
3,6,8	0	28	9.8
−7, −5, −3	0	28	8.5

CBA/Ca mice (five to six/group) were given 10^7 T-depleted B10.BR BM cells on day 0. Three injections of CD4 and CD8 mAbs were given and B10.BR skin was grafted on days as indicated relative to BMT.

However, the time during which mAbs were given seemed very critical for tolerance induction. As is shown in Table II, the same course of mAb therapy completed 3 d before or initiated 3 d after BMT failed to facilitate tolerance; all the mice so treated rejected their donor skin in a secondary set fashion (Table II).

Is the Depletion of Both the CD4 and CD8 Subsets Essential? Recently in a different tolerance system it has been observed that $F(ab')_2$ fragments of CD4 mAbs were sufficient for tolerance induction to human gamma globulin (HGG) (12) and rat Ig (18). In these cases, depletion of $CD4^+$ cells was not required for the induction of tolerance. To establish whether depletion was essential in the present model, we tested whether $F(ab')_2$ fragments, given at high doses daily for 1 wk, could also create an environment permissive for development of BM-induced tolerance. In this experiment only one of the CD4 or CD8 mAb fragments of each synergistic pair was administered. This made it possible to use the partner antibody for monitoring depletion in the "fragment"treated groups. B10.BR BM cells were given to CBA mice together either with complete antibodies as above, $F(ab')_2$ fragments of one mAb plus intact mAbs to the complementary antigens or both CD4 and CD8 mAb $F(ab')_2$. 3 wk after the last mAb injection, all animals and control groups were

TABLE III

Host T Depletion and Tolerance Induction

mAb treatment*	Percent cells‡		Survival of donor skin
	$CD4^+$	$CD8^+$	*MST*
CD4Ig + CD8Ig	<1	<1	>120
CD4Ig + CD8 $F(ab')_2$	<1	21.1	>120
CD4 $F(ab')_2$ + CD8Ig	40.5	<1	8.7
CD4 $F(ab')_2$	35.8	11.2	9.2
CD8 $F(ab')_2$	32.3	10.9	8.6
CD4 Ig	<1	20.3	11.5
CD8 Ig	42.2	<1	8.9

* CBA/Ca mice ($n = 6$) were given 10^7/mouse B10.BR BMCs on day 0. Intact antibodies (Ig) were given as synergistic pairs, 1 mg/mouse in three doses on day 0, 2, and 4. CD4 $F(ab')_2$ was made from YTS 191.1 and CD8 from YTS 169.4. A total of 6 mg/mouse $F(ab')_2$ was given in six injections over 1 wk.

‡ Blood smears were made 3 d after the last mAb injection and stained with immunofluorescence by respective mAbs for different epitopes of CD4 and CD8 molecules.

TABLE IV

Difference of Rat IgG2a and IgG2b mAbs on Cell Depletion and Tolerance Induction

Treatment	PBL lymphocytes						Graft survival
	CD4+		CD8+		Thy-1+		
	%	*MFI*	%	*MFI*	%	*MFI*	*MST*
IgG2a CD4 IgG2b CD8	31.5	86	1.3	157	52.5	607	>120
IgG2a CD4 IgG2a CD8	26.4	86	12.5	76	56.7	503	12
IgG2b CD4 IgG2a CD8	1.7	78	15.3	115	19.8	552	>120
IgG2b CD4 IgG2b CD8	4.3	121	6.5	129	3.5	193	>120
PBS	39.8	157	12.5	185	47.0	430	9.8

Normal CBA mice ($n = 6$) were given 15 d mAb treatment (0.5 mg total mAb/day) as indicated and 10^7 B10.BR BM cells at the beginning of treatment. 5 d after the last mAb injection, mice from each group were bled and PBL lymphocytes analyzed by flow cytometry. Numbers given are percentage of positive cells (mean of fluorescence intensity [MFI]). 3 wk later they were grafted with B10.BR tail skin.

grafted with B10.BR skin. The results are given in Table III. Surprisingly the CD8 mAb can be wholly replaced by $F(ab')_2$ fragments when accompanied by intact CD4 mAbs. However, mice given CD4 $F(ab')_2$ rejected donor test skin irrespective of the nature of CD8 mAb offered.

An alternative way of treating with CD4 and CD8 mAbs without incurring cell depletion is to use nonlytic rat IgG2a mAbs (Qin, S., et al., manuscript in preparation). In Table IV it can be seen that injection of these mAbs resulted in antigenic modulation but not depletion of the relevant subsets. As with the rIgG2b fragments, the rIgG2a CD8 mAb could contribute to tolerance if combined with a rIgG2b CD4 mAb. Remarkably, the rIgG2a CD4 mAb could also enable tolerance if combined with a rIgG2b CD8 mAb. However, the combination of rIgG2a CD4 and CD8 mAbs was surprisingly ineffective.

Alloreactive T Cells Must Be Removed from the Marrow/Spleen Inoculum to Guarantee Tolerance. The tolerogenicity of the B10.BR BM seemed to be impaired by the presence of donor T cells. This is shown in Fig. 3 where only 4 of 11 (27%) CBA/Ca recipients

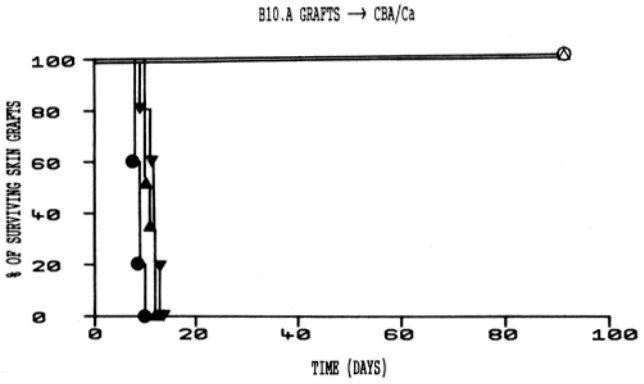

FIGURE 3. Transplantation tolerance achieved in MHC class I and minor mismatches. CBA/Ca mice ($n = 6$) were antibody treated as above: 2×10^7 B10.A BM on the first day of mAb treatment. B10.A, B10.BR and BALB/c skin grafts were transplanted 3 wk later. MST: B10.A (○) >90 d; B10.BR (△) >90 d; BALB/c (▼) 12.1 d. Control (mAb only ▲) 11.5 d; BM only (●) 9.1 d.

were tolerized by nonpurged marrow/spleen inocula. In contrast, purged marrow/spleen produced tolerance in all recipients. Equivalent transplants from (CBA × B10.BR)F_1 donors were able to induce tolerance whether or not T cells had been purged. Similar results were obtained in two consecutive experiments. As will be demonstrated below, the nontolerant animals showed no evidence of donor chimerism. We can only assume that our mAb treatment of recipient was marginal and that some donor alloreactive cells were spared. We have not investigated whether the same would hold true for other strain combinations.

Extending Tolerance to Class I MHC plus Multiple Minor Differences. The original description of transplantation tolerance in the neonate (1) used the strain combinations (CBA × A)F_1 into CBA (H-2^{a+k}→H-2^k). We have similarly found that tolerance for B10.A into CBA/Ca (H-2^a→H-2^k: class I + multiple minor differences) can also be achieved in the adult using the CD4 and CD8 mAbs to help B10.A marrow engraftment (Fig. 4). It is interesting to note that the mice transplanted with B10.A marrow also became tolerant to B10.BR grafts. This suggests that minors were being reprocessed as previously documented in fetal liver radiation chimeras (19).

Failure to Tolerize Over Complete MHC + Minor Differences. Table V shows results of an experiment in which we attempted to induce tolerance of BALB/c mice to B10.BR. BALB/c mice received a standard course of mAb treatment and the accompanying marrow graft from B10.BR donors. 3 wk later these mice were grafted with skin from B10.BR and B10.D2 donors. B10.BR skin was rejected rapidly (MST 10 d) by mAb + BM-treated mice, demonstrating that no tolerance had been induced in this combination. Interestingly, B10.D2 skin was accepted for MST of 56 d by the same mice. Furthermore, they all rejected BALB/k skin, suggesting that there was tolerance to B10 minors in association with H-2^d but not to H-2^k alloantigens. A similar result was obtained when CBA/Ca mice were transplanted with BALB/c marrow under cover of CD4/CD8 mAbs; they became poorly responsive to BALB/k

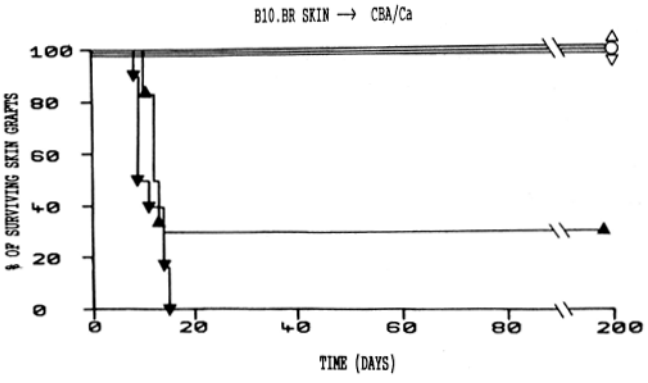

FIGURE 4. The presence of alloresponsive T cells in the marrow graft interfered with tolerance induction. CBA/Ca mice simultaneously receiving CD4 and CD8 mAb therapy were injected with marrow and spleen cells from normal B10.BR (▲, n = 11), T-depleted B10.BR (○, n = 6), normal (CBA × B10.BR)F_1 (△, n = 6), or T-depleted (CBA × B10.BR)F_1 (▽, n = 6). 3 wk after BMT, B10.BR skin was grafted. All grafts survived >16 wk except for mice given B10.BR marrow without T depletion, in which 7 of 11 (73%) grafts were rejected (MST: 10 d, versus (CBA × B10.BR)F_1 group $p \leq 0.002$). A control group received mAbs but no cells (▼, n = 6), and these rejected B10.BR grafts with an MST of 14 d.

TABLE V
Split Tolerance to H-2 and Minor Antigens

Host	Treatment			Graft survival
	mAb	BM	Skin	
				MST
BALB/c	CD4 + CD8	B10.BR	B10.BR	9.8
BALB/c	CD4 + CD8	B10.BR	B10.D2	56
BALB/c	CD4 + CD8	B10.BR	BALB/k	11.2
BALB/c	CD4 + CD8	None	B10.BR	10.2
BALB/c	CD4 + CD8	None	B10.D2	13.0
BALB/c	CD4 + CD8	None	BALB/k	11.4

Normal BALB/c mice ($n = 6$) were injected with CD4 and CD8 synergistic mAb pairs, 1 mg in total in three doses. 10^7 B10.BR BM were given intravenously after the first antibody injection. Skin grafts were performed 3 wk after the last antibody injection.

skin but rejected BALB/c grafts (our unpublished data). In both cases the unresponsive state to donor minors associated with "self-MHC" was not permanent. Most likely, this reflects the disappearance of processable "minor" antigen that follows rejection of donor marrow. Presumably, tolerance waned as the level of tolerogen diminished. These data suggest that whatever the mechanism of marrow rejection in unirradiated MHC-mismatched combinations, there seems to be two fundamentally different and independent immunological phenomena operating after the marrow infusion. One produces priming to donor MHC antigens and the other results in tolerance (albeit shortlived) to donor minors when presented on host-type MHC.

The Lack of Specific Alloreactivity In Vitro of Lymphocytes from Tolerant Animals. The state of tolerance at the level of graft acceptance was reflected in the failure of recipient cells to demonstrate alloreactivity towards tolerizing antigens in vitro. Spleen cells from tolerant mice (B10.BR into CBA/Ca) remained unresponsive when primed in vivo then stimulated in vitro with B10.BR cells while they responded normally to third-party alloantigens (Table VI). Interestingly, spleen cells from tolerant animals

TABLE VI
In Vitro Specific Unresponsiveness of Spleen Cells from CBA Mice Rendered Tolerant by BMT and mAb Therapy

Responder	Stimulator*	Proliferation‡	CML§
Tolerant CBA‖	B10.BR	979 (1.0)	0
	BALB/c	6,004 (1.1)	41 (9.7)
	Normal CBA	632 (1.1)	ND
	None	1,196 (1.2)	ND
Primed CBA	B10.BR	4,353 (1.5)	34 (5.6)
	BALB/c	7,629 (1.1)	28 (8.4)
	Tolerant CBA	5,188 (1.1)	ND
	None	1,161 (1.2)	ND

* 3,000 rad irradiated spleen cells.
‡ Count per minute of IUdR uptake (SD of geometric means).
§ Cell-mediated lympholysis. Figures are percentage of specific ^{51}Cr release at E/T ratio of 40:1 (SD of arithmetic means).
‖ Mice rendered tolerant as in legend to Fig. 2, holding perfect B10.BR skin for >100 d.

TABLE VII
Donor Ig Chimerism after BMT

Treatment*		Percent of chimerism (mean ± SD)‡		
Donor	Recipient	1 wk	4 wk	8 wk
T-depleted B10.BR	T-depleted CBA	0.3 ± 0.2	2.0 ± 0.5	2.1 ± 0.6
Normal B10.BR	T-depleted CBA	0.4 ± 0.2	1.5 ± 0.7	0.4 ± 0.4§
T-depleted (CBAxB10.BR)F_1	T-depleted CBA	0.3 ± 0.2	4.7 ± 2.8	5.6 ± 2.8
Normal (CBAxB10.BR)F_1	T-depleted CBA	0.2 ± 0.2	4.7 ± 2.8	3.2 ± 1.3
T-depleted B10.BR	normal CBA	0	0	0

* Animals were treated as in Fig. 4 legend.
‡ Donor IgG2a chimerism in recipients ($n = 6$) detected by hemagglutination inhibition analyses.
§ Individual results: 0, 0, 0.4, 0.8, 0.8; the first three mice had rejected their skin grafts. Compared with T-depleted group, $p \leq 0.05$.

could stimulate the primed CBA cells. This implies that donor-type antigens were present within the tolerant animals some 120 d after induction of tolerance.

Chimerism Is Necessary for Tolerance. In the multiple minor mismatch combinations that we have used there is no simple and sensitive way to type the majority of hemopoietic cells. We therefore sampled the Ig allotype as a marker of donor B cell chimerism. This analysis was based on the different expression of IgG2a allotypes between black strains of mice (Igh1b) and CBA/Ca mice (Igh1a). In Table VII we document such chimerism for the experimental groups described in Fig. 4. 1 wk after BMT we could detect donor-type IgG2a in the sera of all tolerized mice. This reached peak levels at 2 wk (2–5% of total serum IgG2a) and remained steady thereafter. All animals that maintained their grafts permanently exhibited prolonged B cell chimerism, although in some, donor allotype fell to below detectable levels (<0.2%) after 200 d (not shown). In contrast, we have never been able, in B10.BR→CBA/Ca combination, to detect donor-type Ig in animals that had received marrow without the mAb cocktail. However, we have found a combination, B10.BR→(CBA × BALB/c)F_1, where chimerism was maintained despite skin graft rejection (Table VIII). To distinguish if this was due to the presence of skin-specific antigens or a failure to completely tolerize recipients to antigens well expressed on the skin, an extra sublethal dose of irradiation was given to permit the development of a higher level of chimerism. Mice that underwent such treatment developed 4–5% donor Ig chimerism and then accepted donor skin.

TABLE VIII
Summary of Donor Ig Chimerism and Skin Graft Survival

Donor	Recipient	Treatment	Percent chimerism* (mean ± SD)	Graft survival‡ (MST days)
B10.BR§	CBA	CD4 + CD8	3.2 ± 1.1	>200
B10.A	CBA	CD4 + CD8	2.5 ± 0.4	>200
B10.BR	(CBAxBALB/c)F_1	CD4 + CD8	2.1 ± 0.3	12.3
B10.BR	(CBAxBALB/c)F_1	CD4 + CD8 + TBI‖	5.8 ± 1.2	>60

* Igh1 chimerism analyzed 4 wk after BMT.
‡ Skin grafts performed 3 wk after mAb treatment.
§ $1\text{–}2\times10^7$ T-depleted BM cells given at the time of mAb treatment.
‖ Recipients were given 150 rad in addition to mAbs before BMT.

TABLE IX

T Cell Chimerism after BMT and mAb Treatment

Animals	Treatment	Percent Thy-1.1 (donor type)	Percent Thy-1.2 (host type)
CBA/Ca	mAb + AKR/J BM	3.5 ± 0.5	11.9 ± 1.6
CBA/Ca	mAb	1.7 ± 0.4	13.8 ± 2.7
CBA/Ca	AKR/J BM	1.7 ± 0.7	28.5 ± 0.5
AKR/J	none	31 ± 0.6	1.1 ± 0.1

CBA mice ($n = 6$) were treated with 1 mg/mouse CD4 and CD8 mAbs and 10^7 T-depleted AKR/J BM cells were given. 8 wk later, the phenotype of the recipient PBL was determined by flowcytometry.

Recipient T Cells Become Tolerant. The fact that we had ablated recipient T cells in most of the experiments meant that there was a possibility that the new T cells would be wholly donor derived. If that were the case then we would not be able to claim tolerance induction in recipient T cells. To permit measurements of T cell chimerism in this model of tolerance we extended the studies to follow the consequences of AKR/J marrow grafts into CBA/Ca. In addition to minor transplantation antigen incompatibilities, these strains differ in Thy-1 allotypes. This allowed for measurement of chimerism in the T lineage. AKR/J marrow tolerized CBA/Ca mice (skin graft survival >200 d) as expected (data not shown). From 4 wk after BMT, recipient mice were bled and peripheral mononuclear cells were stained with Thy-1 allelic-specific mAbs. Table IX demonstrates that from 8 wk after BMT, although donor-type Thy-1$^+$ cells are detectable in the tolerant mice, the large majority of T cells are of recipient type.

T Cell Receptor Expression in Tolerant Mice. Recently, a number of reports have shown that the expression of certain TCR β chains correlates with the reactivity to Mls antigens (16, 20, 21). Thus, Vβ6$^+$ mature T cells are absent in Mls^a strain of mice, whereas they constitute 10–15% of lymph node cells in Mls^b mice. Since lymphocytes from Mls^b animals can initiate a strong proliferation response against Mls^a

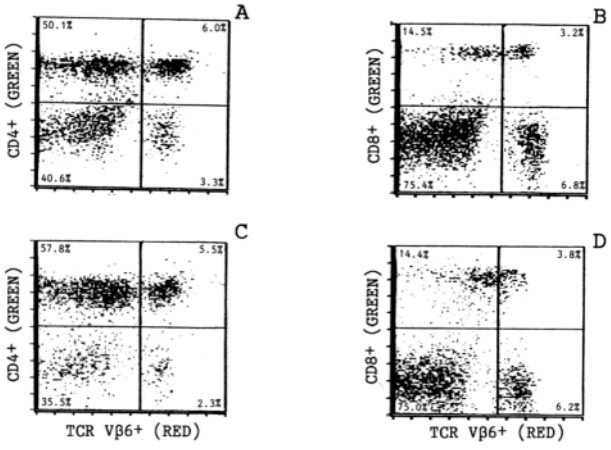

FIGURE 5. Vβ6$^+$ T cells are not deleted from MLS^b mice tolerant of MLS^a grafts. Mouse lymph node cells were double stained with YTS 191.1 (CD4) or YTS 169.4 (CD8) and biotinylated 44-22-1 (TCR Vβ6), followed by FITC-NORIG 7.16 (anti-rat IgG2b) and streptavidin-phycoerythrin. The percentage of each positive population is given in respective regions. (*A, B*) CBA mouse received mAb treatment only; (*C, D*) CBA/Ca mice that had been tolerized with AKR/J BM and CD4+CD8 mAb injection, carrying AKR skin for >120 d. The same reagents stained <1% Vβ6$^+$ cells in control AKR/J mice (not shown). Data represent one of four animals tested, all with similar staining patterns.

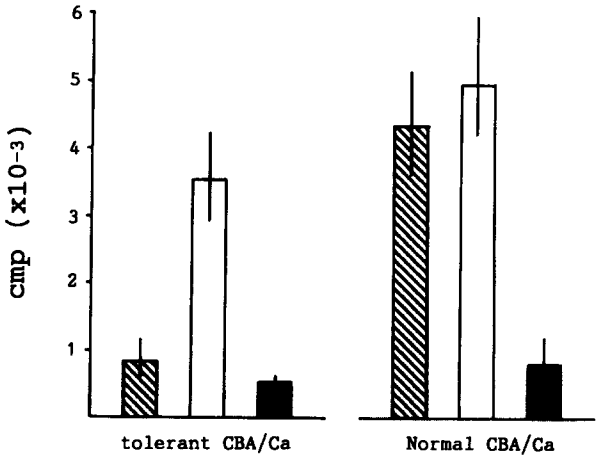

FIGURE 6. In vitro unresponsiveness of CBA mouse spleen cells tolerant to AKR. 5×10^5 spleen cells from mice described in Fig. 5 were incubated with 5×10^5 3,000-rad irradiated AKR (*hatched bar*) or BALB/c (*open bar*) spleen cells. Proliferation was measured by day 4 IUdR incoperation. Negative controls (*solid bar*) were responder cells incubated with medium only.

cells, it has been postulated that TCR Vβ6 may react with Mls^a antigen. It follows that in Mls^a mice the lack of Vβ6$^+$ cells is due to clonal deletion during thymocyte maturation. In the present study, we measured the expression of Vβ6 chain in CBA/Ca (Mls^b) recipients rendered tolerant of AKR/J (Mls^a), carrying AKR skin for >120 d. Lymph node cells from tolerant mice were stained with an anti-Vβ6 mAb 44-22-1 (reference 16) together with mAbs to CD4 or CD8 antigens for two-color flow cytometry. Much to our surprise, these animals had normal levels of Vβ6$^+$ lymph node cells (Fig. 5) and spleen cells (not shown). The percentages of CD4 and CD8 population among these cells were similar to age-matched control mice given mAb treatment only (Fig. 5). However, spleen cells from these tolerant mice were unresponsive in vitro to AKR/J stimulation as measured by proliferation analysis (Fig. 6), and T cell chimerism was consistently detectable (Table IX).

Discussion

We have documented here a number of examples where transplantation tolerance could be achieved in adult animals by introduction of allogeneic BM. In these examples, alloreactivity (graft-vs.-host and host-vs.-graft) had been prevented by injection of CD4 and CD8 mAbs. There are obvious and probably plausible analogies between these findings and the model of classical transplantation tolerance in the neonate. The neonatal mouse lacks immunocompetent peripheral T cells at birth and arguably our use of mAbs also recreated in the adult a "tolerance permissive environment." Our findings have been based on studies using donor-recipient combinations differing in multiple minor transplantation antigen differences and in the case of B10.A onto CBA/Ca, a further class I MHC (H-2D) difference. However, the same regime did not allow us to achieve tolerance in complete MHC (+ minors) mismatched combinations (e.g., BALB/c into CBA or B10.BR into BALB/c). In the past we had to include some level of irradiation (600 rad) in addition to CD4 and CD8 mAbs to guarantee successful marrow engraftment and tolerance in complete H-2 plus minor mismatched situation. With this level of irradiation nearly all the white cells that regenerated were of donor type (22).

The development of chimerism and tolerance was dependent on the prevention

of alloreactivity in both the donor and recipient T cell populations. The presence of T cells in the B10.BR, but not the (B10.BR × CBA/CA)F_1, marrow/spleen inoculum resulted in far fewer CBA/Ca recipients becoming tolerant. It was possible that although the recipients were treated with the CD4 and CD8 mAbs, this was insufficient to adequately cope with all the donor T cell alloreactivity. A similar phenomenon of T cell interference has also been observed in ALS + BM-induced unresponsiveness (23). There are two possible explanations for how T cells interfered with engraftment. The first is that donor alloreactive cells were inhibitory to donor hemopoiesis necessary to establish chimerism. The second is that donor alloreactivity somehow helped residual host lymphocytes to resist or reject the donor stem cells. The available data do not distinguish between the two possibilities. This finding seems to contradict previous data that suggested a role of donor T cells in helping engraftment rather than hindering it (24). However, in those studies, host resistance and hemopoiesis had been further compromised by prior irradiation. We are presently investigating to what extent these findings are applicable to other strain combinations.

We observed that both the recipient CD4 and CD8 subsets were independently capable of resisting B10.BR donor marrow and that both had to be controlled to ensure tolerance in the B10.BR into CBA/Ca combination. Surprisingly the CD8 subset could be "controlled" with rIgG2b $F(ab')_2$ CD8 mAb fragments or a nondepleting rIgG2a mAb, suggesting that depletion of this subset was not essential for tolerance. The failure of tolerance with $F(ab')_2$ fragments of rIgG2b CD4 must have been due to the fact that CD4 mAb fragments had simply not been used in sufficient quantity. Surprisingly, the combination of nondepleting CD4 and CD8 rIgG2a mAbs was unable to create tolerance, although tolerance was possible when either mAb was combined with the complementary rIgG2b mAbs. Notwithstanding this, the importance of these findings is the demonstration that both mature CD4 and CD8 cells are tolerizable without depletion. In a different model of tolerance to HGG and rat IgG, we and others have observed that $F(ab')_2$ fragments of rIgG2b or intact rIgG2a CD4 mAbs were similarly tolerance permissive for CD4 cells (12, 18). Nondepleting LFA-1 mAbs could also create a tolerogenic enviroment where concomitant administration of HGG resulted in long-term tolerance to that antigen (12). The inescapable conclusion of these and other studies (25) is that peripheral CD4 and CD8 cells may still have the choice of turning OFF as well as ON. The outcome would seem to be affected by blockade of CD4, CD8, LFA-1, and perhaps other surface molecules e.g., IL-2 receptors. Perhaps these molecules are crucial to the signaling that determines ON or OFF, or alternatively, when T cells recovering from mAb blockade of these functional molecules encounter antigens in the absence of a helpful influence, the confrontation with antigen may lead to tolerance.

Natural and neonatal tolerance to Mls^a antigens has been reported to be associated with deletion of $V\beta6^+$ cells (16, 20, 26) However, Fowlkes et al. (27) have shown that prolonged administration of CD4 mAb prevented the $V\beta6^+$ cell deletion at the $CD4^+CD8^+$ precursor stage. Our studies have looked at $V\beta6$ expression many weeks after tolerance induction with mAb therapy, and have not revealed depletion of $V\beta6$ cells, although these CBA/Ca (Mls^b) mice showed unresponsiveness to AKR/J (Mls^a) antigens. This finding is not compatible with clonal deletion unless the $V\beta6$ cells we detected had been derived from a cell population that was

Mls[a] nonreactive. On the other hand, these data may suggest that functional anergy may also contribute to a tolerant state, as has been reported for the B cell lineage (28).

Traditionally a major feature of marrow transplantation has been the need to create "space" for a successful graft (29). The "space" concept arose from the need to irradiate or administer myelotoxic agents to achieve successful engraftment. In this study it would appear that no new "space" had to be created for donor marrow to engraft and to establish tolerance to donor antigens. Chimerism of T and B lymphocytes could be achieved with the use of only CD4 and CD8 mAbs that would spare hemopoietic stem cells.

We can categorize the circumstances in which the BM + mAb protocol failed to achieve tolerance into two groups; those where chimerism was established and those where it was not. Those lacking chimerism we consider as examples of residual resistance/rejection. For strong MHC differences our present capacity to control alloreactivity in vivo with mAbs alone may be limited. There are well-documented examples of resistance mechanisms other than CD4 and CD8 cells that can prevent marrow engraftment (30). These may be controlled by the use of additional mAbs or irradiation. Those situations where chimerism is insufficient to guarantee tolerance to skin grafts may represent instances where a tissue (skin)-specific antigen is simply insufficiently represented in the small percentage of chimeric donor hemopoietic cells. The creation of physical space might permit accumulation of sufficient levels of that "minor" antigen to reach tolerogenic thresholds. As tolerance to minors is MHC restricted (31–33), there is a requirement that minors must be presented on appropriate APC for tolerance to occur. As is the case for any other protein, minor antigens can be processed and represented on other cells for tolerance. Our studies have reinforced these findings (i.e., tolerance can be mediated by donor minors processed and presented on host MHC); and they suggest an explanation of the "tissue-specific antigen" phenomenon. Simply put, the chance of a particular tissue-specific minor to tolerize T cells is increased the more APCs there are available to process and present it. In the normal situation, the hemopoietic system is 100% host and 100% of skin is host. Therefore, maximal opportunity for processing and presentation for tolerance exists. In neonatal tolerance or in the present model of adult mAb-facilitated tolerance, only a fraction of the hemopoietic system is donor type, so tolerance to all minor antigens may not always be possible. Irradiation of the recipient would then allow donor cells to accumulate to levels sufficient for tolerance induction.

Although it seems easy to induce tolerance to minor class I antigens with the present mAb therapy, tolerance across full MHC incompatibility is still difficult without irradiation. Our recent work has shown that the inclusion of an anti-LFA-1 mAb into this cocktail allows tolerance with chimerism in complete H-2 mismatch combination with only 300 rad of irradiation (Benjamin, R., et al., manuscript in preparation). It may be that the addition of yet other mAbs may make it feasible to control rejection to produce tolerance without any need to use irradiation or myelotoxic drugs. This would have obvious benefits for clinical practice.

Summary

Transplantation tolerance across histoincompatibilities in multiple non-H-2 minors (B10.BR into CBA/Ca) and "minor" plus H-2D (B10.A into CBA/Ca) antigens has

been achieved successfully by combined adult bone marrow transplantation and treatment with CD4 and CD8 mAbs. The tolerant state was confirmed by permanent acceptance of donor strain skin grafts, and in vitro unresponsiveness to donor cells. Tolerance was associated with partial donor chimerism to various degrees. Tolerance to minor transplantation antigens induced in this manner was restricted to recipient-type MHC. The possibility was raised that tolerance resulted, at least in part, from clonal anergy rather than deletion.

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