MIXED CHIMERISM AND PERMANENT SPECIFIC TRANSPLANTATION TOLERANCE INDUCED BY A NONLETHAL PREPARATIVE REGIMEN

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The induction of stable mixed allogeneic chimerism as an approach to transplantation tolerance has recently been reviewed in detail (1). Two murine models for the induction of such stable mixed chimerism involve total lymphoid irradiation (TLI)[1] followed by infusion of allogeneic bone marrow (BM) (2) and lethal whole body irradiation (WBI) followed by reconstitution with a mixture of T cell-depleted (TCD) syngeneic and allogeneic BM (3, 4). Both of these regimens have been shown to produce long-term mixed lymphohematopoietic chimerism and specific tolerance to donor skin grafts, and in both cases the resulting mixed chimeras have been shown to be otherwise immunocompetent (4, 5).

One major drawback, however, to the clinical use of these approaches to the induction of transplant tolerance has been the excessive toxicity of both irradiation regimens required for the establishment of mixed chimeras. The recent report by Cobbold et al. (6) that treatment of mice with anti-T cell mAbs could promote allogeneic marrow engraftment was therefore of both theoretical and practical interest. However, the chimerism achieved by these authors was transient unless relatively high doses of whole body irradiation (600 and 850 rad) were administered in addition to the mAb treatment. Animals prepared in this fashion demonstrated fully allogeneic reconstitution rather than mixed chimerism (6), and the immunocompetence of these chimeras was not examined.

In present study we have attempted to reproduce and extend these results. We have found that treatment of mice with anti-T cell mAbs leads to profound peripheral T cell depletion but fails to deplete mature T cells from the thymus. We therefore reasoned that the requirement for high dose whole body irradiation to achieve stable chimerism in the studies by Cobbold et al. might have been due to the need for removal of mature thymic T cells. While thymectomy might overcome this problem, it would also leave an animal without thymic stromal elements capable of educating new T cells from host and donor stem cell populations. We therefore attempted selective thymic irradiation (TI) to deplete thymic T cells. We report here that this procedure

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[1] *Abbreviations used in this paper:* BM, bone marrow, BMT, bone marrow transplantation; GVHD, graft-versus-host disease; RAMB, rabbit anti-mouse brain; TCD, T cell-depleted; TI, thymic irradiation; TLI, total lymphoid irradiation; WBI, whole body irradiation.

permits stable mixed allogeneic chimerism to develop in animals following a low dose (300 rad) of whole body irradiation and mAb treatment.

Materials and Methods

Animals. 8–16-wk-old mice of strains C57BL/10Sn (B10), C57BL/6 (B6), B10.D2/nSn (B10.D2), and B10.BR/SgSn (B10.BR) were purchased from The Jackson Laboratory, Bar Harbor, ME. B6.PL-Thy-1^a (B6-Thy-1.1) mice (7) were obtained from the Frederick Cancer Research Facility, Frederick, MD. All mice were maintained in a specific pathogen-free environment.

Experimental Protocol. The two mAbs used for in vivo treatment were GK1.5, a rat anti-mouse CD4 mAb (8), and 2.43, a rat anti-mouse CD8 mAb (9). Both GK1.5 and 2.43 hybridoma lines were obtained from the American Type Culture Collection, Rockville, MD. These mAbs were purified from ascites prepared in nude mice by 50% ammonium sulfate precipitation followed by filtration on an Ultrogel AcA 34 column (IBF Biotechnics, Sevage, MD). Recipient B10 or B6 mice were treated with both purified mAbs (500 μg each) by intraperitoneal injection 5–6 d before bone marrow transplantation (BMT). Control animals were treated with PBS or with 1 mg of purified rat IgG (Organon Teknika Corp., West Chester, PA). On the day of BMT, WBI (120 rad per minute) was administered using a ^{137}Cs source and selective thymic irradiation (63 rad per minute) was performed using a therapeutic x-ray machine (Philips Electronic Instruments, Inc., Mahwah, NJ). For thymic irradiation, mice were anesthetized and positioned under a lead shield from which a hole had been cut in order to selectively irradiate the region of the thymus. Donor B10.D2 or B6-Thy-1.1 bone marrow was harvested from the long bones as previously described (4) and was administered intravenously in a 1-ml volume (10–15 $\times$ 10^6 cells). T cell depletion of B6-Thy-1.1 BM cells was accomplished by treatment with rabbit anti-mouse brain (RAMB) serum and guinea pig complement (10) before administration to B6 recipients.

T Cell Depletion Analysis. Mice treated with mAbs alone were tested for the level of T cell depletion by flow cytometry analysis on a FACS II (Becton Dickinson & Co., Mountain View, CA). 5 d after mAb treatment PBMC, spleen cells, and thymocytes were stained directly with floureseinated anti-Thy-1.2 (Becton Dickinson Immunocytometry System, Mountain View, CA) or indirectly with GK1.5 (0.5 μg) plus 2.43 (0.5 μg) followed by fluoresceinated mouse mAbs to rat Ig light chain (MAR18.5, Becton Dickinson & Co.).

Characterization of Chimeras. Transplanted mice were tested for the level of allogeneic donor T and non-T cells by two-color flow cytometry analysis as previously described (11). PBL were stained with fluoresceinated anti-Thy-1.2 (green) and with biotinylated anti-H-2^d (34-2-12) (12) antibodies plus Texas Red Streptavidin (Bethesda Research Laboratories, Gaithersburg, MD) (red). Two-color data were displayed as contour diagrams with logarithmically increasing intensities of green (FITC) fluorescence plotted on the x axis versus logarithmically increasing intensities of red (Texas-Red) fluorescence on the y axis. Chimerism in the Thy-1 congenic model (B6-Thy1.1$\rightarrow$B6) was determined by staining donor T cells with fluoresceinated anti-Thy-1.1 (Bioproducts for Science INC., Indianapolis, IN) (green) and host T cells with biotinylated anti-Thy-1.2 plus Texas Red Streptavidin (red). Nonspecific staining was assessed with irrelevant mAbs (fluoresceinated or biotinylated anti-Leu-4; Becton Dickinson & Co.).

Evaluation of Specific Tolerance. Specific tolerance was assessed by comparing the survival time of donor skin grafts (B10.D2) with the survival time of third-party skin grafts (B10.BR). Skin grafts were performed as previously described (4) between 6–8 wk after BMT by the method of Billingham (13), using full-thickness tail skin grafted to the lateral thorax. Grafts were examined daily in the first month and then every 2 d for signs of rejection. Rejection was considered complete when no viable tissue was detectable by visual inspection.

Results

Minimal Host Conditioning Required to Attain Allogeneic Engraftment. Initial studies were performed to determine the level of T cell depletion obtainable after in-

traperitoneal administration of anti-T cell mAbs. A time course study showed that maximal depletion of T cells in the peripheral tissues after administration of 500 μg each of GK1.5 and 2.43 occurred within 5 d after treatment (Table I). However, this same mAb treatment failed to deplete T cells from the thymus. The total number of thymic cells did not significantly change, and thymic T cells were found to be coated with the injected rat antibodies but not eliminated, as indicated by staining with the MAR-FITC reagent. As shown in Table II *A*, group 1, this treatment regimen was found to be inadequate to permit engraftment of allogeneic BM, as donor cell chimerism was not detectable 2 wk after BMT.

Two additional treatments were therefore added to the mAbs in order to determine the minimal requirements for obtaining detectable chimerism 2 wk after administration of allogeneic bone marrow: (*a*) Administration of a low dose of WBI (300 rad), the lowest dose previously shown to permit transient engraftment after mAb treatment (6); and (*b*) administration of thymic irradiation to a total dose of 1,000 rad, the dose of WBI previously used for production of stable mixed allogeneic chimeras in this laboratory (4). The results of these studies are shown in Table II. As seen from these data, none of the three conditioning treatments alone (Table II *A*, groups 1-3) was sufficient to achieve chimerism in animals examined 2 wk after BM administration. Of the three possible combinations of these treatments taken two at a time, only mAbs plus the 300-rad WBI were sufficient to produce chimerism (Table II *A*, group 6). However, as shown for one representative animal in Fig. 1, this chimerism was transient, with subsequent loss of detectable donor cells and without induction of tolerance. 3 of 3 such animals grafted with donor skin rejected their grafts within 14 d, as did unmanipulated animals. On the other hand, more consistent stable engraftment was achieved by addition of 700 rad selective thymic irradiation to the mAb and 300 rad WBI treatments (Table II *A*, group 7). In the first set of experiments, 4 of 10 animals treated with this regimen developed long-term, stable mixed chimerism, as shown for one representative animal in Fig. 2. T cells of both host and donor origin developed by day 32 and chimerism in both T and non-T cells persisted >150 d. Stable mixed chimerism was accompanied by induction of long-term tolerance to donor B10.D2 skin grafts (grafted 39

TABLE I

T Cell Level 5 d after In Vivo Administration of GK1.5 plus 2.43

		Percent positive cells			
	Cell source:	Blood	Spleen	Thymus	
In vivo treatment	Staining mAbs:	Anti-Thy-1.2-FITC		GK1.5 + 2.43 + MAR-FITC*	MAR-FITC
PBS‡		28	21	99	2
Rat IgG‡		31	22	99	2
GK1.5 + 2.43§		4	7	98	43
GK1.5 + 2.43§		2	4	97	70

* Fluoresceinated mouse anti-rat mAb.

‡ Control mice were treated 5 d before analysis with PBS or 1 mg nonspecific rat IgG.

§ Individual mice were injected 5 d before analysis with 500 μg each of GK1.5 (anti-CD4) and 2.43 (anti-CD8).

TABLE II

Host Preparative Regimens Attempted for Allogeneic BM Engraftment

Group	Host conditioning* mAbs	300-rad WBI	TI (rad)	Number of mice with donor cells in blood 2 wk after BMT (percent donor cells)	Number of mice with surviving donor skin grafts (>100 d)‡
A					
1	+	-	—	0/7	—
2	-	+	—	0/7	—
3	-	-	1,000	0/3	—
4	+	-	1,000	0/7	—
5	-	+	700	0/3	—
6	+	+	—	7/8 (4-53)	0/3
7	+	+	700	10/12 (13-71)	4/10
B					
1	+	+	—	9/10 (19-50)	4/10
2	+	+	700	10/10 (31-79)	10/10

B10 mice receiving the different conditioning regimens were infused with 15 $\times 10^6$ unmanipulated B10.D2 BM cells. Chimerism in peripheral blood was analyzed by flow cytometry using staining with anti-donor H-2 antibodies (34-2-12, anti-D^d). Transplanted animals were grafted between 6-8 wk after BMT with donor skin grafts (B10.D2).

(*A*) Data represent summary of several experiments.

(*B*) Data from a single experiment performed after improved thymic irradiation protocol.

* Intraperitoneal injection of GK1.5 (anti-CD4) plus 2.43 (anti-CD8), 5-6 d before BMT. WBI and TI administered on day of BMT.

‡ All animals with long-term survival of donor skin grafts demonstrated stable mixed chimerism (>150 d).

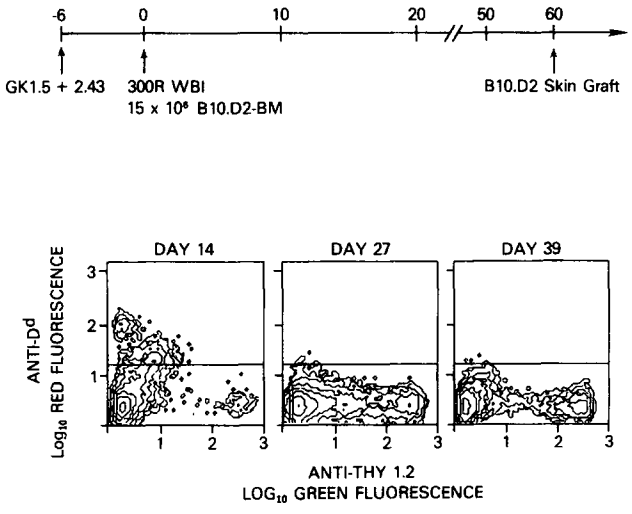

FIGURE 1. Development of transient chimerism without tolerance after administration of mAbs, 300-rad WBI, and allogeneic BM cells. (*Top*) Experimental manipulations were performed on indicated days. (*Bottom*) Two-color immunofluorescence profile of PBL from a typical chimera prepared according to this protocol. Contour plots after staining with anti-Thy-1.2, staining all T cells (green fluorescence, horizontal axis), and anti-D^d, staining all cells of B10.D2 donor origin (red fluorescence, vertical axis) are shown. Cuts of contour plots were done such that they included 97-99% of positive control cells (B10.D2) staining with anti-D^d and <2% of negative cells (B10). In all cases staining with irrelevant mAb was done, and was less than or equal to staining of B10 cells.

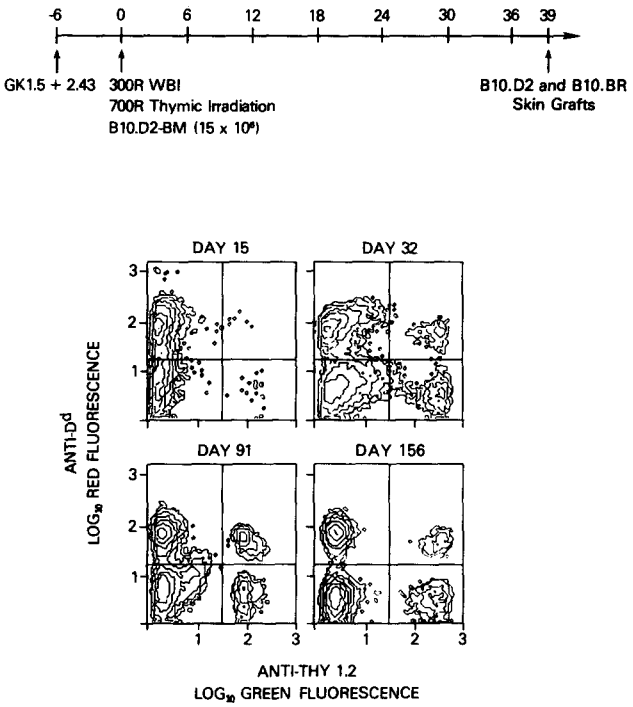

FIGURE 2. Development of mixed chimerism and specific tolerance after administration of mAbs, 300-rad WBI, 700-rad thymic irradiation, and allogeneic BM cells. (*Top*) Experimental manipulations were performed on indicated days. (*Bottom*) Two-color immuno-fluorescence profiles of PBL from typical chimeras prepared according to this protocol. Contour plots after staining with anti-Thy-1.2 mAb, staining all T cells (green fluorescence, horizontal axis), and anti-D^d mAb, staining all cells of B10.D2 donor origin (red fluorescence, vertical axis) are shown. Cuts of contour plots on the x-axis were done such that they included <0.2% of cells staining with irrelevant antibodies. Cuts of contour plots on the y-axis were done such that they included 97–99% of positive control cells (B10.D2) staining with anti-D^d and <2% of negative cells (B10). In all cases staining with irrelevant mAb was done, and was less than or equal to staining of B10 cells.

d after BMT), which remained intact for >100 d. Control B10 animals that did not receive B10.D2 BM rejected such grafts within 14 d (data not shown).

In an attempt to increase the incidence of stable mixed chimerism achieved by this combination treatment regimen (i.e., mAbs, 300-rad WBI, and 700 rad thymic irradiation), a more reliable system for assuring exposure of thymus to irradiation was devised using chest markings. After such treatment, as shown in Table II *B*, 10 of 10 animals developed stable mixed chimerism persisting >150 d. Stable chimerism in each case was associated with tolerance to donor skin grafts. In this experiment, 4 of 10 control animals without thymic irradiation also developed permanent chimerism and long-term survival of donor skin grafts (Table II *B*). In contrast to the first set of experiments, however, the rejection of donor cells in most of the nontolerized mice was slow and proceeded after donor skin grafts were already rejected. Thus, in all experiments thymic irradiation markedly increased the percentage of animals developing stable chimerism and tolerance. Pooling the results of all experiments, engraftment increased from 30% (4/13) without TI to 70% (14/20) with TI, which is statistically significant ($p < 0.05$ by χ^2 analysis).

Specificity of Transplant Tolerance. The specificity of tolerance in the mixed chimeric animals was determined by comparing their responses to donor skin grafts (B10.D2) and to third-party skin grafts (B10.BR). All chimeras that demonstrated long-term survival of donor (B10.D2) skin grafts were fully reactive to third-party (B10.BR) skin grafts (as shown in Fig. 3 for animals in Table II *B*), rejecting such grafts at

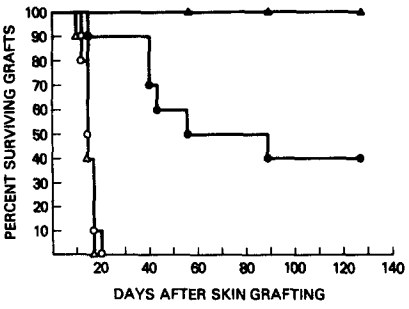

FIGURE 3. Specific tolerance to donor alloantigens in mixed chimeric animals. B10 (H-2^b) mice were treated with mAbs GK1.5 (anti-CD4) plus 2.43 (anti-CD8) and 300-rad WBI with (▲, △) or without (●, ○) 700-rad thymic irradiation. Pretreated animals were infused with 15 × 10^6 B10.D2 (H-2^d) BM cells and received donor B10.D2 (H-2^d) skin grafts (▲, ●) and third-party B10.BR (H-2^k) skin grafts (△, ○) 7 wk later.

the same time as did control animals not receiving B10.D2 BM (data not shown). Such long-term, specific tolerance was found exclusively in animals with stable mixed chimerism.

Host Survival without Bone Marrow Administration. As demonstrated in Fig. 4, the combination host conditioning regimen that produced long-term, mixed allogeneic chimerism was in itself nonlethal, in contrast to the WBI regimen previously used to achieve mixed allogeneic chimerism without mAb treatment (4, 14). Animals treated by the combined protocol but not receiving marrow remained healthy and gained weight at a rate similar to untreated control animals.

Minimal Host Conditioning for Syngeneic BM Engraftment. In an attempt to determine whether 300 rad WBI was required in order to over come residual host alloresistance after mAb treatment, or to create "room" for engraftment of injected BM, we studied the minimal conditions required for BM engraftment in the absence of alloreactivity. For this purpose, Thy-1 congenic mice (B6-Thy-1.1→B6) that differ only in the allelic form of Thy-1 antigen on T cells were used. Thy-1 alleles are not known to generate transplant reactions in mice (Mobraaten, L. E., The Jackson Laboratory, Bar Harbor, ME; personal communication). In this experiment T cells were depleted from donor marrow so that only T cell chimerism arising from stem cells or precursor cells in the marrow would be detected. As shown in Table III, conditioning of syngeneic hosts (B6) with 300-rad WBI was necessary and sufficient for engraftment of T cell depleted (TCD) B6-Thy-1.1 BM. In contrast, treatment

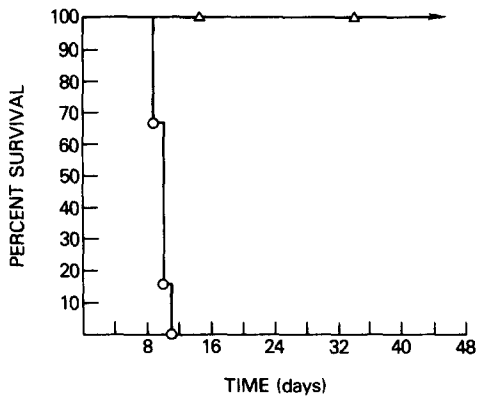

FIGURE 4. Mice treated with the complete regimen without BM transplant demonstrated long-term survival. Survival of B10 mice (4) that received mAbs, 300-rad WBI and 700-rad thymic irradiation (△) without BM is compared with the survival of lethally irradiated (1,025 rad) mice (6) not receiving BM (○).

TABLE III

Minimal Host Conditioning for BM Engraftment in Thy-1 Congenic Strain Combination

Host conditioning*		Percent donor Thy-1.1$^+$ cells in peripheral blood T cells
mAbs	300 rad WBI	
–	–	0‡
+	–	0
–	+	10-34
+	+	36-51

B6 recipients (two to three mice in each group), either untreated or treated with mAbs and/or 300-rad WBI, were infused with 10^7 TCD B6-Thy-1.1 BM. T cell chimerism was assessed 4 mo after BMT by staining with fluoresceinated anti-Thy-1.1 and biotinylated anti-Thy-1.2.

* B6 recipients were injected with 500 μg of both GK1.5 (anti-CD4) and 2.43 (anti-CD8) mAbs 5 d before BMT. 300-rad WBI was administered on the day of BMT.

‡ No detectable staining by fluoresceinated anti-Thy-1.1 above background staining of negative control animal (B6) cells.

with anti-CD4 plus anti-CD8 alone did not permit engraftment of TCD B6-Thy-1.1 marrow. The possibility that mAbs prevented engraftment by depleting donor T cells (either residual T cells in the BM inocula or newly developing donor T cells in the host) was unlikely, since administration of mAbs with 300-rad did not prevent engraftment. Thus 300 rad WBI was essential for engraftment even in a system in which rejection of donor cells by the host was unlikely.

Discussion

The use of mAbs directed against T cell subsets in vivo represents a powerful addition to the preparative regimens now available for the induction of immunological nonresponsiveness. One major limitation of this approach, however, is the potential for relevant cells to escape destruction either because they are inaccessible to the administered antibodies, or because they are coated by the antibodies but not depleted. Possible causes for failure to deplete coated target cells include modulation of surface antigen and/or inaccessibility of the cells to mechanisms leading to elimination of coated cells in vivo. Our findings suggest that the previous failure by Cobbold et al. to achieve permanent allogeneic engraftment after 300-rad WBI, in vivo anti-T cell mAb treatment, and administration of allogeneic bone marrow (6) may have been due to sparing of T cells in the thymus. The present studies demonstrate that anti-CD4 plus anti-CD8 mAbs lead to effective depletion of peripheral blood and spleen T cells by day 5, but that thymic T cells are only coated with mAbs and not eliminated. This observation is consistent with previous reports demonstrating the inability of anti-CD4 (15) or anti-Thy-1.1 (16) to eliminate thymocytes.

The addition of 700 rad selective thymic irradiation to the preparative regimen was attempted in order to raise the total dose of thymic irradiation to 1,000 rad, a dose that permits allogeneic engraftment when administered as WBI without mAb treatment (14). This additional radiation led to long-term, stable chimerism and specific tolerance in 4 of 10 animals in the first set of experiments and in 10 of 10 animals for which the improved TI protocol was applied. These data strongly sug-

gest that the transience of engraftment in previous protocols was due to the inability to eliminate thymic T cells by treatment with mAbs. The importance of eliminating T cells from the thymus has also been demonstrated in the TLI model (2), in which the thymus as well as the peripheral lymphoid tissues must be included in the irradiation field in order to achieve the full immunosuppressive effect of the irradiation treatment.

In all experiments in the present study, thymic irradiation increased the incidence of stable chimerism and transplantation tolerance. In one experiment, however, 40% of animals not receiving thymic irradiation also developed specific tolerance. This difference between experiments may have been due to age-related changes in the state of thymic maturation. Investigations of the effects of thymic irradiation in animals of different ages are now in progress.

It is also clear from our data that mAb treatment plus thymic irradiation alone was insufficient to permit engraftment. Despite the fact that the dose of WBI used (300 rad) was far too low to ablate host resistance to alloengraftment by itself (Table II), this dose appeared to be sufficient to allow alloengraftment to proceed when combined with mAb treatment and thymic irradiation. This dose of WBI was also found to be essential for the engraftment of BM from otherwise syngeneic, Thy-1 congenic donors, from which BM did not engraft in unmanipulated hosts or after in vivo mAb treatment alone. These data are consistent with the hypothesis that "room" must be made for engraftment of self-renewing cell populations in order for chimerism to be achieved. We cannot rule out the possibility that in the allogeneic model, 300-rad WBI is also necessary to deplete a residual host cell population responsible for host resistance.

Using this preparative regimen there appeared to be no GVHD despite the administration of non-TCD allogeneic BM. This might be due to elimination of mature T cells from the donor BM inocula by residual anti-CD4 and anti-CD8 mAbs that persist in the host blood for more than 2 wk after treatment (data not shown). Such elimination of donor T cells would not be expected to impair engraftment since TCD allogeneic BM also engrafts using the preparative regimen described here (17).

Animals prepared by the combined regimen (mAb treatment, 300-rad WBI, and 700-rad thymic irradiation) demonstrated mixed chimerism rather than the fully allogeneic reconstitution achieved with the higher dose (600 or 850 rad) of WBI and mAb treatment used by Cobbold et al. (6). Mixed allogeneic chimerism as an approach to the induction of donor specific transplantation tolerance has practical and theoretical advantages over fully allogeneic chimerism (1). The autologous elements provide a safeguard against aplasia in case of alloengraftment failure, as demonstrated in this study by the excellent survival of mice receiving the preparative regimen without allogeneic BM. In addition, autologous cells provide a source of host type accessory cells for presentation of antigen to new T cells educated in the host thymus, thus contributing to the immunocompetence of the chimeric animals (3, 4).

Summary

The use of allogeneic bone marrow transplantation as a means of inducing donor-specific tolerance across MHC barriers could provide an immunologically specific conditioning regimen for organ transplantation. However, a major limitation to this approach is the toxicity of whole body irradiation as currently used to abrogate host

resistance and permit marrow engraftment. The present study describes methodology for abrogating host resistance and permitting marrow engraftment without lethal irradiation. Our preparative protocol involves administration of anti-CD4 and anti-CD8 mAbs in vivo, 300-rad WBI, 700-rad thymic irradiation, and unmanipulated fully MHC-disparate bone marrow. B10 mice prepared by this regimen developed stable mixed lymphohematopoetic chimerism without any clinical evidence of graft-vs.-host disease. Engraftment was accompanied by induction of specific tolerance to donor skin grafts (B10.D2), while third-party skin grafts (B10.BR) were promptly rejected. Mice treated with the complete regimen without bone marrow transplantation appeared healthy and enjoyed long-term survival. This study therefore demonstrates that stable mixed chimerism with donor-specific tolerance can be induced across an MHC barrier after a nonlethal preparative regimen, without clinical GVHD and without the risk of aplasia.

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