Specific Tolerance Induction Across a Xenogeneic Barrier: Production of Mixed Rat/Mouse Lymphohematopoietic Chimeras Using a Nonlethal Preparative Regimen

By Yedida Sharabi, Ivan Aksentijevich, Thoralf M. Sundt III, David H. Sachs, and Megan Sykes

From the Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Summary

The development of safe methods for inducing donor-specific tolerance across xenogeneic barriers could potentially relieve the critical shortage of allograft donors that currently limits the applicability of organ transplantation. We report here that such tolerance can be induced in a xenogeneic combination (rat → mouse) using a nonmyeloablative and nonlethal preparative regimen. Successful induction of chimerism and donor-specific transplantation tolerance required pretreatment of recipients with monoclonal antibodies (mAbs) against NK1.1, Thy-1.2, CD4 and CD8, followed by administration of 3 Gy whole body radiation (WBI), 7 Gy thymic irradiation, and infusion of T cell–depleted rat bone marrow cells (BMC). Rat cells appeared among peripheral blood lymphocytes (PBL) of such recipients by 2–3 wk, and rat T cells by 2–5 wk following bone marrow transplantation (BMT). Donor-type rat skin grafts placed 4 mo after BMT were accepted, while simultaneously placed non–donor-type rat skin grafts were promptly rejected. In addition to its clinical potential, the ability to induce donor-specific tolerance across xenogeneic barriers using such a nonlethal preparative regimen provides a valuable model for the study of mechanisms of xenogeneic transplantation tolerance.

An inadequate supply of allogeneic donors has become the major limitation to the rapidly advancing field of clinical organ transplantation. Transplantation of organs across species barriers could potentially provide a solution to this problem. Although the use of lethal irradiation and bone marrow transplantation (BMT)[1] has been shown to induce transplantation tolerance across xenogeneic barriers in animal models (1–5), the clinical applicability of such an approach is limited by the toxicity of the preparative regimen. It will therefore be essential to develop less toxic methods for inducing xenogeneic chimerism if this approach is to be useful clinically. Previous work from this laboratory has demonstrated that chimerism and transplantation tolerance can be induced across complete allogeneic MHC barriers in mice using a nonlethal preparative regimen, in which anti-CD4 and anti-CD8 mAbs are used instead of lethal irradiation to eliminate mature T cells from the host. Following this mAb treatment, relatively nontoxic whole body irradiation (WBI) at 3 Gy plus 7 Gy thymic irradiation were sufficient to permit engraftment of allogeneic bone marrow (6). Attempts to extend this model to a xenogeneic rat/mouse strain combination were not successful using the same nonlethal preparative regimen that was effective in allogeneic combinations. However, addition of antibodies against host NK cells and Thy-1$^+$ cells to the preparative regimen led to successful induction of xenogeneic chimerism and specific transplantation tolerance. Although the level of chimerism diminished gradually over time, specific acceptance of donor skin grafts was durable, indicating that specific transplantation tolerance across a xenogeneic barrier can be induced using a nonlethal preparative regimen.

[1] *Abbreviations used in this paper:* BMC, bone marrow cells; BMT, bone marrow transplantation; FCM, flow cytometry; LCA, leukocyte common antigen; TCD, T cell–depleted; TI, thymic irradiation; TRA, Texas red streptavidin; WBI, whole-body irradiation.

Materials and Methods

Animals. Male C57BL/10SnJ (B10) and B10.D2/nSn (B10.D2) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Male Wistar-Furth (WF) and Fisher 344 (F344) rats were purchased from Frederick Cancer Research Facility, Frederick, MD. All animals were maintained in a specific pathogen-free facility.

Conditioning and BMT. B10 recipients (12–20 wk old) received mAbs intraperitoneally on days −6 and −1. Doses of each mAb were as follows: 0.1 ml of GK1.5 (7) (rat anti-mouse CD4) ascites (cytotoxic titer 1:64,000); 0.1 ml of 2.43 (8) (rat anti-mouse CD8) ascites (cytotoxic titer 1:80,000); 500 μg 30-H12 (9) (rat anti-mouse

Thy-1.2), purified from ascites by 50% ammonium sulfate precipitation followed by filtration on an Ultragel AcA 34 column and; 400 μg of PK136 (murine anti-NK1.1 mAb [10]) 50% ammonium sulfate precipitated. On day 0, 3 Gy WBI and 7 Gy selective thymic irradiation (TI) were administered to mAb-treated animals, as previously described (6). Bone marrow cells (BMC) were administered intravenously on the same day, as described (6). Animals received 60×10^6 rat (F344) BMC, which had been T cell–depleted (TCD) using mAb R1-3B3 (11) (anti-CD5) followed by two cycles of rabbit complement (Cedarlane Laboratories, Ontario, Canada). Control animals received 15×10^6 allogeneic B10.D2 BMC, which were T cell–depleted using anti-CD4 plus anti-CD8 plus anti-Thy-1.2 mAbs and rabbit complement, as described (12). Depletion of rat marrow was evaluated by flow cytometry (FCM) analysis after staining with fluoresceinated anti-CD2 (OX-34; Bioproducts for Science, Indianapolis, IN). Fewer than 0.7% $CD2^+$ T cells remained after depletion.

Phenotyping of BMT Recipients. Rat PBL, thymocyte and BMC chimerism were evaluated by staining with FITC-conjugated mAb OX-1 (Bioproducts for Science), which recognizes a rat leukocyte common antigen (LCA) expressed on all rat leukocytes. Rat T cells were detected using FITC-conjugated anti-rat CD5 mAb OX-19 (Bioproducts for Science). In control recipients of allogeneic BMT, donor-type chimerism was tested by staining with biotinylated mAb 34-2-12 (anti-D^d) (13). For detection of host-type cells, biotinylated anti-K^b mAb 5F1 (14) was used. For detection of murine T cells, biotinylated anti-Thy-1.2 mAb (Becton Dickinson & Co., Mountain View, CA) was used. Expression of Thy-1.1, but not of the Thy-1.2 allele, has been detected on some types of rat cells (15). For red fluorescence (detected on FACS II), incubation with biotinylated antibody was followed by incubation with Texas red streptavidin (TRA; Bethesda Research Laboratories, Bethesda, MD). For orange fluorescence (detected on FACSCAN), incubation with biotinylated antibody was followed by incubation with phycoerythrin-streptavidin (PEA). FITC-conjugated and biotinylated mAb Leu-4 (Becton Dickinson & Co.) were used as nonstaining irrelevant antibodies for green and red or orange staining, respectively.

FCM Analysis. Two-color FCM was performed as described (16) using a FACS II (Becton Dickinson) or a FACSCAN (Becton Dickinson). Contour plots were generated as described (17). For calculation from contour plots of the percentage of cells staining with FITC-labeled mAbs, the percentage of cells in each green-positive rectangle after staining with Leu-4–FITC was subtracted from the percentage of cells staining with the FITC-conjugated test antibody in the same rectangle; for determination of the percentage of cells staining with biotinylated mAbs, the percentage of cells in each red or orange-positive rectangle after staining with Leu-4–biotin was subtracted from the percentage of cells staining with the biotinylated test antibody in the same rectangle.

Skin Grafting. Full thickness skin grafting was performed according to a modification of the method of Billingham, as described (18). Grafts were evaluated daily, and were considered to be rejected when less than 10% of the original graft was detectable.

Statistical Analysis. Skin graft survival probability was determined using the censored data technique of Kaplan-Meier, and statistical significance was determined using the method of Wilcoxon and Breslow. All statistical results are expressed as P values, and values less than 0.05 are considered to be significant.

Results

mAb Requirements for Induction of Mixed Rat/Mouse Chimerism. Groups of recipient B10 mice were prepared as indicated in Table 1. Xenogeneic PBL chimerism was evaluated 2–3 wk following BMT by staining with mAb recognizing all rat leukocytes (OX-1) or rat T cells (OX-19). Rat PBL chimerism was not detectable in any of 12 animals pretreated with anti-CD4 plus anti-CD8 mAbs (Table 1, Exp. 1). In contrast, this regimen permitted the development of allogeneic chimerism in 9 of 12 control recipients of B10.D2 BMC in the same experiment (Table 1, Exp. 1), similar to previous results (6).

Since NK cells can mediate alloresistance (19–23), we considered the possibility that host NK cells might be preventing engraftment of xenogeneic marrow. We therefore examined the effect of depleting host NK cells by adding anti-NK1.1 mAb to the preparative regimen. PBL chimerism was produced in two of six such recipients (Table 1, Exp. 2, Group 3). This result, however, was not significantly different from that achieved in animals pretreated with anti-CD4 plus anti-CD8 alone in this experiment, in which one of four animals demonstrated PBL chimerism 2–3 wk after BMT (Table 1, Exp. 2, Group 1). Chimerism was not achieved in any of six animals pretreated with anti-NK1.1 mAb without anti-CD4 or anti-CD8 (data not shown).

Since our regimen of treatment with anti-CD4 plus anti-CD8 mAbs is associated with persistence of a small residual population of $Thy\text{-}1^+$ $CD4^-$, $CD8^-$ cells (Sharabi, Y., and D. H. Sachs, unpublished data), we evaluated the effect on xenogeneic marrow engraftment of eliminating this population by pre-treating recipients with large amounts of anti-Thy-1.2 mAb. As shown in Table 1 (Exp. 2, Group 2), significant, but low (6–10% of PBL) levels of rat PBL chimerism were detectable in six of six recipients pretreated with anti-Thy-1.2 mAb in addition to anti-CD4 plus anti-CD8.

In the same experiment, we also evaluated the effect of combined pretreatment with anti-Thy-1.2, anti-NK1.1, anti-CD4, plus anti-CD8 mAbs on engraftment of rat BMC. As is shown in Table 1 (Exp. 2, Group 4), pretreatment with this combination of mAbs was associated with engraftment of rat cells in 10 of 10 recipients. The levels of rat cell repopulation at this early time point (2–3 wk after BMT) were approximately twice as high as those in animals treated with anti-Thy-1.2, anti-CD4 and anti-CD8 (Group 2). The time course of the two-color FCM profile produced by PBL from a representative animal in this group is shown in Fig. 1. It was apparent from these data that optimal early engraftment of rat marrow was attained using a combination of mAbs against CD4, CD8, Thy-1.2, and NK1. Similar results were obtained in a repeat experiment (data not shown).

Time Course of Rat PBL Repopulation. The percentage of rat PBL repopulation in recipients of various mAb pretreatments was further evaluated at later time points. As shown in Fig. 2, the percentage of rat cells declined gradually over time in all chimeric recipients originally pretreated with anti-CD4, anti-CD8, plus anti-Thy-1.2, with or without anti-NK1.1 mAb. By ~6 mo after BMT, only one of five survivors of the three mAb pretreatment and two of six survivors of the four mAb regimen contained >1% rat PBL (Fig. 2). Of the two chimeric animals pretreated with anti-CD4, anti-CD8, and anti-NK1.1 mAbs, one animal lost its chimerism

Table 1. *Engraftment of TCD Rat BMC in Mice Prepared Using a Nonlethal Regimen*

Exp.	Group	mAb treatment*	Donor‡	Fraction of chimeric animals§ (percent donor cells) 2–3 wk	5 wk
1	1	Anti-CD4 + anti-CD8	B10.D2	9/12 (14–81)	
	2	Anti-CD4 + anti-CD8	F344	0/12	
2	1	Anti-CD4 + anti-CD8	F344	1/4 (3)	0/4
	2	Anti-CD4 + anti-CD8 + anti-Thy-1.2	F344	6/6 (6–10)	5/6 (1–9)
	3	Anti-CD4 + anti-CD8 + anti-NK1.1	F344	2/6 (3–14)	1/6 (4)
	4	Anti-CD4 + anti-CD8 + anti-Thy-1.2 + anti-NK1.1	F344	10/10 (9–24, 66‖)	9/9 (1–9, 77‖)

* B10 recipients were injected with mAbs on day −6 and day −1, followed by administration of 300 rad WBI, 700 rad TI, and BM cells on day 0.

‡ 15 × 10^6 B10.D2 BM, T cell depleted with anti-CD4 (GK1.5[15]), anti-CD8 (2.43[16]) and anti-Thy-1.2 (30-H12[17]) plus complement or 60 × 10^6 F344 BM, T cell depleted with anti-CD5 (R1-3B3[18]) and complement.

§ Xenochimerism was detected by flow cytometry analysis after staining PBL with fluoresceinated anti-rat LCA (OX-1) or with irrelevant mAb, fluoresceinated anti-human CD3 (Leu-4). Percent rat cells was calculated by the formula: Percent rat cells = 100 × {[(% $OX\text{-}1^+$ − % $Leu\text{-}4^+$ cells in transplanted animal) − (% $OX\text{-}1^+$ − % $Leu\text{-}4^+$ cells in B10 control)]/[(% $OX\text{-}1^+$ − % $Leu\text{-}4^+$) in rat control]}. Allochimerism was tested by flow cytometry after staining donor cells with anti-D^d (34-2-12[19]) mAb and percent donor cells was calculated by a similar formula.

‖ One animal in this group showed exceptionally high levels of rat cell engraftment.

by 35 d after BMT, whereas rat cells became undetectable in the PBL of the other animal between 90 and 120 d after BMT (data not shown).

In most chimeric animals in all groups, rat T cells were not detected among PBL tested 2–3 wk after BMT. Among PBL of all mice pretreated with anti-CD4, anti-CD8, anti-NK1.1, and anti-Thy-1.2, rat T cells appeared by 5 wk after BMT, and their percentage continued to increase until 7 wk in most animals (Table 2). The presence of such T cells in one representative animal is illustrated in Fig. 2. By day 90, the percentage of rat T cells had declined significantly in all animals, and continued to decline in parallel with the general decline of rat PBL chimerism (Table 2, Fig. 2). Among six chimeric animals pretreated with anti-Thy-1.2, anti-CD4, and anti-CD8 mAbs without anti-NK1.1, rat T cells appeared among PBL of three mice, and followed a similar time course to that observed in animals pretreated with all four mAbs (Table 2). Rat T cells appeared with a similar time course among PBL of one of two chimeric animals pretreated with anti-CD4, anti-CD8, plus anti-NK1.1 mAbs (data not shown).

Most animals appeared healthy for the duration of the experiment, with no clinical evidence for GVHD. The only exceptional animal was the one outlier recipient in group 4 (Table 1) which demonstrated 66% rat PBL reconstitution by 2 wk after BMT. This animal appeared wasted and ill, and died 7 to 13 wk after BMT. This was also the only animal in which rat T cells were detectable by 2 wk after BMT, consistent with the possibility that GVHD had developed in this recipient.

In general, murine T cells began to appear at the same time as rat T cells, i.e., between 3 and 5 wk after BMT (data not shown).

Rat Cell Repopulation of Other Lymphoid Compartments. To evaluate engraftment of rat cells in lymphoid organs other than PBL, one animal was killed 45 d after BMT, and staining of splenocytes, thymocytes, and BMC was performed. As shown in Fig. 3, $OX\text{-}1^+$ rat leukocytes were detectable among thymocytes and BMC; rat cells were also detectable among splenocytes (data not shown). Only a fraction of the $OX\text{-}1^+$ rat cells in peripheral organs stained with the OX-19 T cell marker, in proportion to the overall percentage of T cells in each organ (see normal rat and mouse controls, Fig. 3); the percentage of $OX\text{-}19^+$ cells among thymocytes was approximately equal to the total percentage of $OX\text{-}1^+$ cells in this organ (Fig. 3).

Induction of Transplantation Tolerance. To determine whether or not recipients were tolerant of donor antigens, donor-type F344 rat skin grafts were placed on all survivors ~120 d after BMT. The results, shown in Fig. 4 (top), demonstrate that animals pre-treated with either anti-CD4 plus anti-CD8 alone ($n = 4$), or with anti-NK1.1 mAb in addition to anti-CD4 plus anti-CD8 ($n = 6$), rejected F344 skin grafts with a similar time course to that demonstrated by normal B10 mice ($n = 4$) ($p > 0.05$). Thus, tolerance was not induced in these mice. In contrast, animals originally pretreated with anti-Thy-1.2, anti-CD4, plus anti-CD8 mAbs ($n = 5$) demonstrated significant prolongation of donor skin graft survival ($p = 0.01$). Skin graft rejection in this group followed a chronic pattern with signs of inflammation apparent by 50 d after grafting in all animals, leading to rejection by 82 d (205 d after BMT). The most striking prolongation of F344 graft survival was observed among recipients originally pretreated with all four mAbs (anti-Thy1.2, anti-NK1.1, anti-CD4, anti-CD8). Five of six animals in this group retained F344 skin grafts in ex-

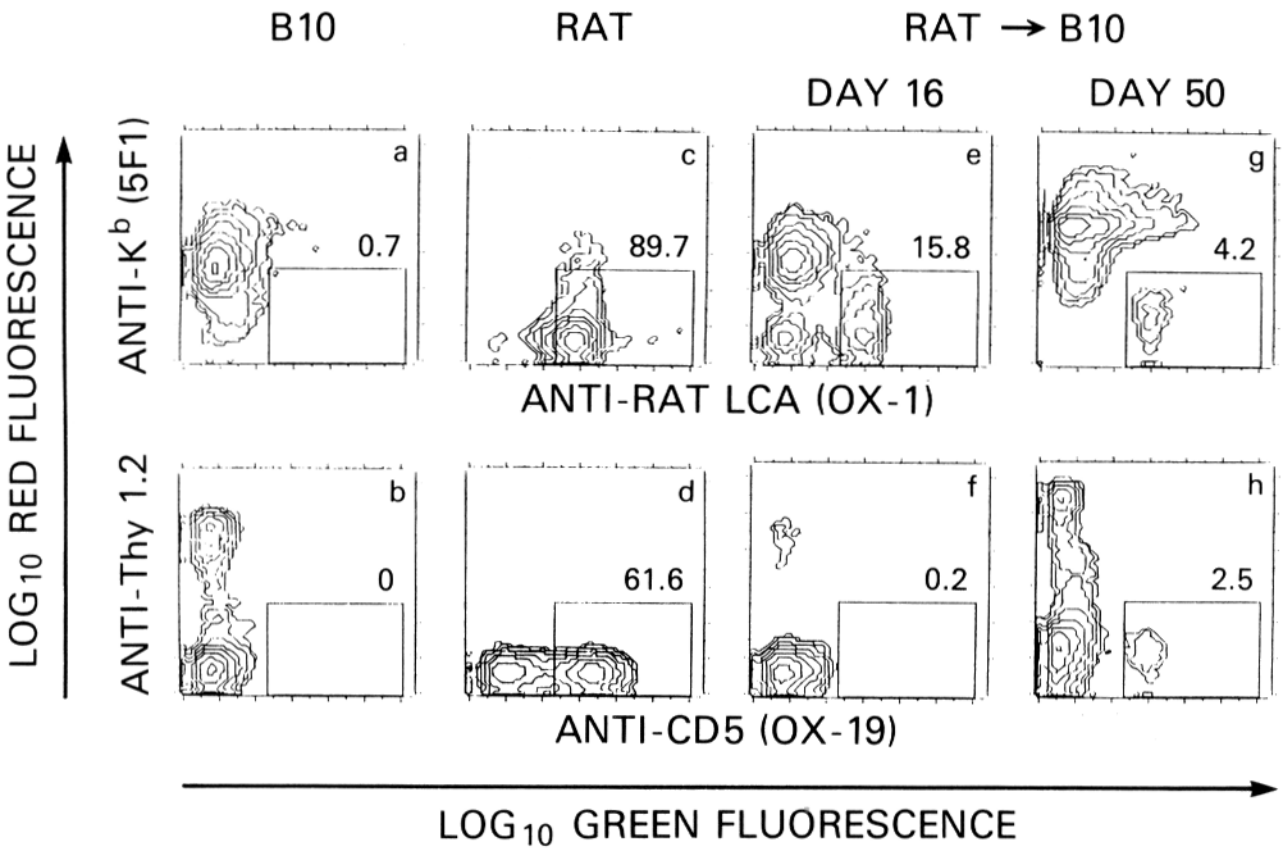

Figure 1. Development of xenochimerism in B10 mice pretreated with anti-CD4, anti-CD8, anti-Thy-1.2 plus anti-NK1.1 mAbs followed by 300 rad WBI, 700 rad TI, and infusion of 60 × 10^6 TCD rat BM. Two-color immunofluorescence profiles of PBL from B10 control (*a* and *b*), rat control (*c* and *d*), and a typical chimera (*e–h*). (*Top*) Contour plots after staining with fluoresceinated anti-rat LCA (OX-1) mAb, which stains all rat leukocytes (green fluorescence, horizontal axis) and biotinylated anti-K^b (5F1) mAb plus TRA, staining all cells of B10 origin (red fluorescence, vertical axis). (*Bottom*) Contour plots after staining with fluoresceinated anti-CD5 (OX-19), which stains rat T cells (green fluorescence, horizontal axis), and biotinylated anti-Thy-1.2 plus TRA, staining murine T cells (red fluorescence, vertical axis). Percentages of cells in each rectangle were determined by subtraction of background staining, as described in Materials and Methods.

cellent condition for more than 110 d. In several animals, episodes of mild inflammation, presumably due to rejection episodes, appeared and subsequently resolved. The prolongation of skin graft survival in this group was highly significant compared with B10 controls ($p = 0.002$).

There was not a complete correlation between skin graft acceptance and PBL chimerism at the time of skin grafting. For example, one animal originally pretreated with all four mAbs no longer displayed significant PBL chimerism by day 90 after BMT, but retained its F344 skin graft in perfect condition by day 220 after BMT. Conversely, one animal pretreated with anti-Thy-1.2, anti-CD4 plus anti-CD8 mAbs still demonstrated rat PBL chimerism by 183 d after BMT, but chronically rejected its F344 skin graft, with complete rejection by

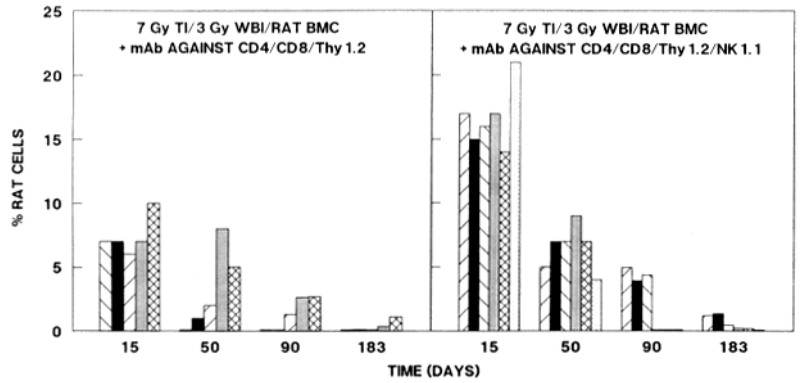

Figure 2. Rat PBL chimerism at various times following BMT. Percentages of OX1$^+$ cells among PBL of individual animals were determined by FCM, as described in Materials and Methods, and calculations were performed as described in the legend to Table 1. Each type of bar represents a single animal at the different time points shown after BMT (horizontal axis). (*Left*) OX1$^+$ cells among PBL of B10 mice pretreated with anti-CD4, anti-CD8, plus anti-Thy-1.2 mAbs; (*right*) OX1$^+$ cells among PBL of B10 mice pretreated with anti-CD4, anti-CD8, anti-Thy-1.2, plus anti-NK1.1 mAbs. All animals received 7 Gy TI plus 3 Gy WBI before infusion of 60 × 10^6 TCD rat BMC.

Table 2. *Rat T Cell Repopulation in PBL of Mice Prepared with a Nonlethal Regimen*

MAb pretreatment	Animal	Weeks‡:	Rat T cells in PBL* 2–3	5	7	26	Skin graft survival
			%				d§
Anti-CD4 + anti-CD8 + anti-Thy-1.2	1		0.4	5.8	4.7	1.2	74
	2		0	0	0	0	18
	3		0	1.3	0.1	0	82
	4		0.1	0	0	0	8
	5		0	2.0	3.4	0.7	74
Anti-CD4 + Anti-CD8 + anti-Thy-1.2 + anti-NK1.1	1		0.2	3.3	4.1	1.3	>110
	2		0.3	3.8	5.8	0.8	28
	3		0.8	3.9	5.8	0.2	>74‖
	4		0.2	3.0	3.0	0.1	>110
	5		0.3	1.7	3.8	0.1	>110
	6		0	1.8	0.3	0	>110

* Determined using anti-CD5 mAb OX19 as described in Materials and Methods.
‡ Weeks after BMT.
§ Days after skin grafting. Grafting with F344 rat skin was performed ~18 wk after BMT.
‖ Animal died 74 d after skin grafting with intact graft.

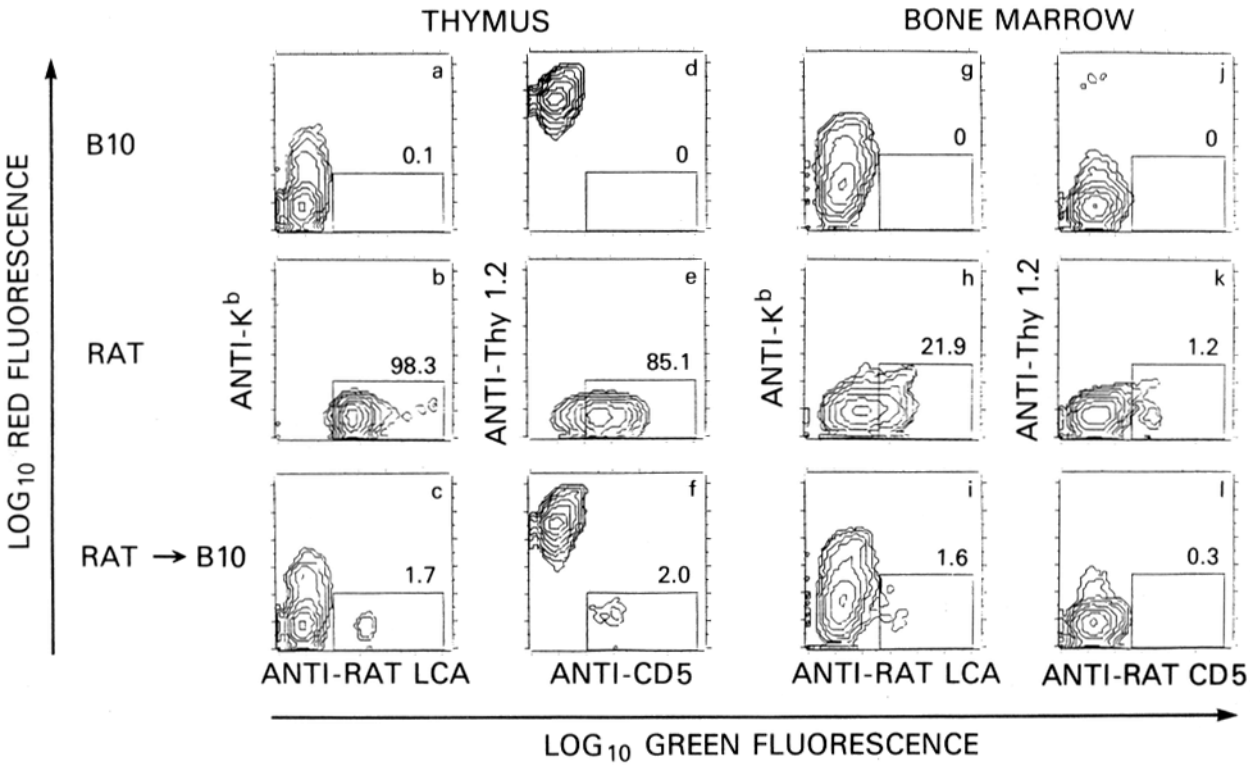

Figure 3. Xenogeneic engraftment in thymus and bone marrow. Two-color immunofluorescence profiles of thymocytes (*a–f*) and BMC (*g–l*) from B10 control (*a*, *d*, *g*, and *j*), rat control (*b*, *e*, *h*, and *k*) and a typical chimera (*c*, *f*, *i*, and *l*) prepared by the modified nonlethal regimen, 45 d after BMT. Data presented as contour plots after staining with fluoresceinated OX1 (anti-rat LCA, staining all rat leukocytes) (*a–c* and *g–i*), or with fluoresceinated OX19 (anti-rat CD5, staining rat T cells) (*d–f* and *j–l*) (green fluorescence, horizontal axis) and biotinylated anti-K^b mAb plus TRA (*a–c* and *g–i*), staining all cells of mouse origin or biotinylated anti-Thy-1.2 mAb plus TRA (*d–f* and *j–l*), staining mouse T cells (red fluorescence, vertical axis).

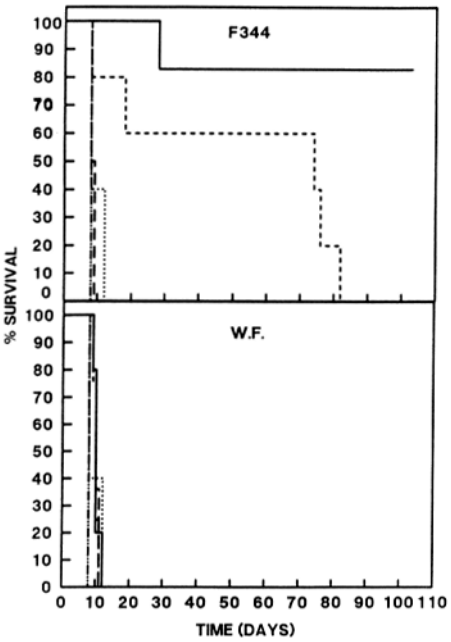

Figure 4. Survival of donor-type (*top*, F344) and non–donor-type (*bottom*, *WF*) rat skin grafts placed 123 d after BMT on recipients of 7 Gy TI, 3 Gy WBI, and 60 × 10^6 TCD F344 BMC, following pretreatment with: (— —) anti-CD4 plus anti-CD8 mAbs; (····) anti-CD4, anti-CD8, plus anti-NK1.1 mAbs; (---) anti-CD4, anti-CD8, plus anti-Thy-1.2 mAbs; (—) anti-CD4, anti-CD8, anti-Thy-1.2, plus anti-NK1.1 mAbs. (—···—) Skin graft survival on untreated B10 controls.

197 d after BMT. Among animals pretreated with anti-Thy-1.2, anti-CD4, and anti-CD8 mAbs, the three animals demonstrating marked prolongation of F344 skin graft survival were the only three animals in this group in which rat T cells appeared among PBL at any time (see Table 2).

Specificity of Transplantation Tolerance. To determine whether or not tolerance to rat skin grafts was donor-specific, a WF, non-donor-type rat skin graft was placed on the opposite side of the thorax at the same time as grafting with F344 (donor-type) skin. As shown in Fig. 4 (*bottom panel*), these grafts were promptly rejected by all groups. Thus, the transplantation tolerance induced in these animals was specific for the rat strain of the donor marrow.

Discussion

Due to the inadequate supply of allogeneic organ donors, it will be essential to overcome xenogeneic transplantation barriers if transplantation is to reach its full potential as a therapeutic modality. While the ability to induce donor-specific tolerance, obviating the need for chronic immunosuppression, would be most desirable, such an approach is hampered by the fact that currently available regimens for inducing allogeneic chimerism are myeloablative and are therefore extremely toxic. We have recently described a method for producing allogeneic chimerism and transplantation tolerance across MHC barriers that specifically targets mature host T cells for elimination, and that therefore has minimal myelotoxicity (6). In the present report, we demonstrate that a modification of this regimen can be used to induce specific transplantation tolerance across xenogeneic barriers. This modification involves administration of antibodies against NK1.1 and Thy-1.2 antigens in addition to the anti-CD4 plus anti-CD8 mAbs used for the induction of allogeneic tolerance. These results imply that, in contrast to alloengraftment, more exhaustive depletion of T cells and depletion of NK cells may be necessary to achieve xenoengraftment. Since some NK cells, including those that are activated (24), as well as NK cell precursors (25), express the Thy-1 marker (24), and a small population of NK cells do not express NK1.1 (10), it is possible that both anti-Thy-1.2 and anti-NK1.1 are required for adequate elimination of NK cells. Lymphokine activated killer (LAK) cells (24), which likewise express Thy-1, may also be involved in xenoresistance. NK cells have been shown to resist engraftment of allogeneic BM grafts in mice (19–23), and might play an even stronger role in resisting xenoengraftment. Our data would also be consistent with a role for Thy-1^+ $CD4^-$, $CD8^-$ T cells, such as those that express a TCR-γ/δ on their surface, in preventing engraftment of xenogeneic marrow.

Although initial levels of chimerism were significantly greater among animals pretreated with anti-NK1.1, anti-Thy-1.2, anti-CD4, and anti-CD8 mAbs, than among recipients of a similar pretreatment regimen without anti-NK1.1, these differences disappeared with time, so that the levels of total PBL and T cell chimerism were not noticeably different between the two groups by the time of skin grafting at 120 d after BMT. The eventual rejection of donor skin grafts by chimeric animals originally pre-treated with anti-Thy-1.2, anti-CD4, plus anti-CD8 mAbs without anti-NK1.1 might therefore reflect the activity of persistent $NK1.1^+$ xenoresistant cells that were not ablated by the preparative regimen. Since the addition of anti-NK1.1 antibody to the pretreatment regimen eliminated such resistance without prolonging the duration of chimerism, this result suggests that nontolerant $NK1.1^+$ cells may participate directly in xenogeneic skin graft rejection, or alternatively, may prevent the development of T cell tolerance without preventing initial engraftment of xenogeneic marrow. It has recently been demonstrated that $NK1.1^+$ TCR-α/β–bearing cells exist in the bone marrow of normal mice (Sykes, M., manuscript submitted for publication), and Yankelevitch et al. have recently demonstrated a role for cells with this phenotype in the rejection of allogeneic bone marrow grafts (20); such cells might be capable of specifically recognizing xenoantigens and participating in skin graft rejection.

The imperfect correlation between long-term chimerism and transplantation tolerance is as yet unexplained; however, the minimal requirements for tolerance induction clearly include both adequate ablation of host resistance and conditions permitting the early development of chimerism. It is possible that, among animals pretreated with all four mAbs, chimerism persists indefinitely in organs that may be critical to the maintenance of tolerance, such as the thymus, similar

to results in neonatally tolerized mice (26). Alternatively, a tolerant T cell repertoire might be formed early after transplantation, and may persist even after rat cell chimerism has disappeared. While evidence suggests that clonal deletion of T cells reactive against histocompatibility antigens borne on BM-derived elements occurs in the thymus, the type of BM-derived cell responsible for such clonal deletion has not been defined (27, 28). The failure to induce tolerance in the absence of persistent peripheral lymphoid chimerism in some allogeneic BMT models (29–31) might reflect incomplete ablation of host immunity rather than a need for persistent chimerism per se in the maintenance of tolerance.

Among animals pretreated with anti-Thy-1, anti-CD4, plus anti-CD8 mAbs without anti-NK1.1, only those in which rat T cells were detectable among PBL showed marked prolongation of donor skin graft survival. This result is consistent with the possibility that donor cells in the thymus are involved in the production of a tolerant T cell repertoire, but that residual xenoresistant cells (possibly NK cells) rejected the skin grafts.

It is possible that donor rat skin grafts on animals pretreated with all four mAbs will be rejected very late after transplantation. In fact, it has previously been hypothesized that the expression of skin-specific antigens on rat skin grafts might preclude their permanent acceptance in animals that are tolerant of rat lymphohematopoietic antigens by in vitro assays (2). Nevertheless, the excellent appearance of most skin grafts at 110 d already exceeds the prolongation that was achieved using a lethal preparative regimen and mixed xenogeneic BMT in the same strain combination (1, 2). In addition, the appearance of these skin grafts is much closer to that of normal skin (including hair growth) than was observed using the previous lethal preparative regimen.

The delayed appearance of rat T cells in the PBL of xenogeneic marrow recipients prepared with anti-Thy-1.2 mAb along with anti-CD4, anti-CD8, 3 Gy WBI and 7 Gy TI, suggests that thymic seeding by rat stem cells had occurred. The possibility that rat stem cells engrafted was further supported by the presence of rat cells in bone marrow and thymus 45 d after BMT (Fig. 3). We cannot, however, rule out the possibility that engraftment of committed progenitor, and not of pluripotent stem cells, occurred, and that the gradual disappearance of rat cells from the circulation reflects a primary failure of pluripotent stem cell engraftment. The development of transplantation tolerance under such circumstances underscores our lack of knowledge regarding the identity of the BMC that are responsible for tolerance induction.

There are other possible explanations for the gradual loss of rat cell chimerism in these animals. For example, it is possible that rat stem cells are at a competitive disadvantage (32) compared with mouse stem cells in a murine environment, and that they don't survive as long or divide as frequently as they would in a homologous environment. Alternatively, rat chimerism may be lost due to an immunologic mechanism. Since the same animals demonstrate transplantation tolerance, it is unlikely that such a mechanism would involve T cells, which are major effectors of skin graft rejection (33). Specific antibody, on the other hand, may be present without causing skin graft rejection (34), and could be responsible for rejection of lymphohematopoietic cells. Arguing against this possibility, however, is the observation that cytotoxic antibody against rat BMC or spleen cells did not appear in the serum of any of the tolerant animals when tested at multiple time points between 15 and 183 d after BMT (Aksentijevich, I., et al., unpublished data). Finally, it is possible that newly developing murine NK cells are not tolerized by the regimen described here, and that such cells effect gradual bone marrow graft rejection.

In summary, we have demonstrated that donor-specific tolerance can be induced across a xenogeneic barrier using a nonmyeloablative preparative regimen. Depletion of recipient NK cells and Thy-1$^+$ cells appears to be necessary for the induction of such tolerance. Tolerant animals demonstrated early lymphopoietic chimerism, including the appearance of donor T cells in peripheral blood. Studies are in progress to further dissect the requirements for tolerance induction in this system. The nonmyeloablative approach used here could potentially bring the induction of donor-specific tolerance to xenogeneic transplantation antigens into the realm of clinical feasibility.

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Address correspondence to Dr. David H. Sachs, NIH, National Cancer Institute, 9000 Rockville Pike, Bldg. 10/4B13, Bethesda, MD 20892.

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