

Cross-species Bone Marrow Transplantation: Evidence for Tolerance Induction, Stem Cell Engraftment, and Maturation of T Lymphocytes in a Xenogeneic Stromal Environment (Rat → Mouse)

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Summary

Transplantation of untreated F344 rat bone marrow into irradiated B10 mouse recipients (non-TCD F344 → B10) to produce fully xenogeneic chimeras resulted in stable xenogeneic lymphoid chimerism, ranging from 82% to 97% rat. Survival of animals was excellent, without evidence for GVH disease. The specificity of tolerance which resulted was highly donor-specific; MHC disparate third party mouse and rat skin grafts were promptly rejected while donor-specific F344 grafts were significantly prolonged (MST >130 days). Multi-lineage rat stem cell-derived progeny including lymphoid cells (T- and B-lymphocytes), myeloid cells, erythrocytes, platelets, and natural killer (NK) cells were present in the fully xenogeneic chimeras up to 7 months after bone marrow transplantation. Immature rat T-lymphocytes matured and acquired the α/β T-cell receptor in the thymus of chimeras in a pattern similar to normal rat controls, suggesting that immature T-lymphocytes of rat origin could interact with the murine xenogeneic thymic stroma to undergo normal maturation and differentiation. This model may be useful to study the mechanisms responsible for the induction and maintenance of donor-specific transplantation tolerance across a species barrier.

Technical advances in solid organ transplantation and the availability of nonspecific immunosuppressive agents have revolutionized the field of solid organ transplantation. Kidneys, livers, hearts, lungs, and pancreases are now transplanted with excellent results (1–4). This progress has resulted in a critical shortage of essential organs of suitable size and match. Many recipients die while awaiting a lifesaving cardiac or hepatic transplant for which there is no substitute life support system. A renewed interest in xenogeneic transplantation has emerged as one potential solution to the critically short supply of solid organs (5). However, transplantation of solid organs across a species barrier has not matched the success of allogeneic combinations, even for closely related species, because rejection is vigorous and not prevented by conventional immunosuppressive agents (5). Transplantation of T cell depleted (TCD)¹ allogeneic bone marrow following lethal irradiation has been previously shown to result in stable stem cell engraftment and donor-specific transplantation tolerance (10–19). Induction of tolerance through transplantation of bone marrow

from one species into another to produce fully xenogeneic chimerism has been suggested as a possible approach to overcome these limitations.

It has long been known that rat bone marrow injected into irradiated mice can significantly prolong survival (6–9). Congdon and Lorenz (1954) reported that survival of irradiated recipient mice was prolonged to 21 days following the single intravenous injection of rat bone marrow cells (6). Using a phosphatase stain for leukocytes, Nowell et al. extend this work to demonstrate that the transplanted bone marrow was probably functioning hematopoietically as evidenced by rat leukocyte production (7). However, longitudinal studies were limited by poor survival of the chimeras, with most chimeras surviving less than 4 weeks following reconstitution (6, 7). Mice which survived long term had erythrocytes and granulocytes of rat origin detectable (7). Similar findings were reported by Ford et al. (8), who demonstrated that irradiated CBA mice reconstituted with Wistar Furth bone marrow cells had detectable rat cells by chromosomal analysis when animals were sacrificed at 5, 11, or 19 days (8). Zaalberg et al. demonstrated that rat skin grafts of donor type were accepted in those mice with detectable rat RBC chimerism (9). However, studies of these mouse recipients of rat bone marrow were limited by the technology available at that time to docu-

¹ Abbreviations used in this paper: CML, cell mediated lympholysis; MLR, mixed lymphocyte reaction proliferative assay; MST, medium survival time; non-TCD, non-T cell depleted; RAMB, rabbit anti-mouse brain; TCD, T cell depleted.

ment engraftment and by extremely poor survival of recipient mice (6–9). In all studies, characterization of the specificity of tolerance induced and multi-lineage production by rat stem cells was not preformed. In fact, there is a paucity of data to indicate whether bone marrow from one species can survive long term and function normally in the stromal environment of another species (5). Such information is critical for future progress in transplantation of xenogeneic bone marrow or solid organs.

We now present evidence for stable rat pluripotent stem cell engraftment and maturation of rat-T-lymphocytes in the xenogeneic stromal environment of normal irradiated B10 mouse recipients after transplantation of untreated rat bone marrow. The specificity of tolerance in these chimeras was highly selective: the tolerant recipient did not see RAT as generic RAT; instead, the tolerance induced was donor MHC strain-specific. Fully xenogeneic chimeras exhibited significant prolongation of donor-strain rat skin grafts with rapid rejection of MHC-disparate third-party rat and mouse skin grafts. This model may be useful to study the mechanism responsible for the induction and maintenance of donor-specific transplantation tolerance across a species barrier. In addition, the kinetics of engraftment of the pluripotent stem cell and time-related regulation of stem-cell-derived lineage production in a xenogeneic environment can be explored.

Materials and Methods

Animals. Six to eight week old male C57BL/10SnJ (B10), B10.BR, and C3H/HeJ (C3H) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Four to eight week old male Fischer 344 (F344) and Wistar Furth (WF) male rats were purchased from the Harlan Sprague Dawley Company. Animals were housed in a specific pathogen-free facility at the Pittsburgh Cancer Institute.

Xenogeneic Reconstitutions (Non-TCD F344 → B10) or (Non-TCD WF → B10). Fully xenogeneically reconstituted animals were prepared by a modification of the method previously described for preparation of mixed allogeneic chimeras (15, 20). Briefly, inbred C57BL/10Sn male recipients were lethally irradiated with a single dose of 950 R from a Cesium source (Nordion). Using sterile technique, bone marrow was flushed with Media 199 (Gibco, Grand Island, New York) containing 50 µl/ml Gentamicin (MEM) from the femurs and tibias of mice and rat donors with a 22 gauge needle. The marrow was mechanically resuspended in MEM by gentle aspiration through an 18 gauge needle and the suspension filtered through sterile nylon mesh. The cells were then pelleted at 1000 rpm for 10 minutes, resuspended, and counted. B10 bone marrow cells were T-cell depleted by treatment with a 1 to 40 dilution of rabbit anti-mouse-brain (RAMB) in MEM (1×10^8 cells/ml) (4°C for 30 minutes) plus guinea pig complement (37°C for 30 minutes) (Gibco, Grand Island, New York) as previously described (5, 12). Cells were washed twice and resuspended in MEM at the appropriate concentration to allow injection of 1 ml per animal. Rat bone marrow was untreated. Recipient animals were reconstituted within 4 to 6 hours following lethal irradiation via the lateral tail veins using a #27 gauge needle. Fully xenogeneically reconstituted animals received 40×10^6 non-TCD F344 (F344 → B10) or Wistar Furth (WF → B10) rat bone marrow cells. Mixed xenogeneically reconstituted animals received 5×10^6 T-cell depleted bio mouse bone marrow cells plus 40×10^6 non-TCD F344 rat

bone marrow cells unless otherwise specified. Radiation controls were prepared to confirm adequacy of the radiation.

Characterization of Chimeras by Flow Cytometry. Recipients were characterized for engraftment with syngeneic and/or xenogeneic donor lymphoid elements using flow cytometry to determine the percentage of peripheral blood lymphocytes (PBL) bearing H-2^b and F344 or WF surface markers (15, 20). Briefly, peripheral blood was collected into heparinized plastic serum vials. After thorough mixing, the suspension was layered over 3 ml of room temperature LSM, (Organon Technica, Kensington, MD) and centrifuged at 23°C (1700 rpm × 30 minutes). The lymphocyte layer was aspirated from the saline-LSM interface and washed with medium. Red blood cells were ACK-lysed (ammonium chloride potassium carbonate lysing buffer, made in our laboratory) and the remaining cells stained with various monoclonal antibodies for 30 minutes at 4°C and counterstained with sandwich when required. Arbitrary levels on log scale were selected based on the inflection point where staining of the control negative population was minimized while retaining maximal numbers of positive cells.

Analyses of splenic and thymic lymphoid cells were performed using a Becton-Dickinson Fluorescence Activated Cell Sorter (FACS II) as previously described (15, 20). Monoclonal antibodies anti-WF and anti-F344-Biotin, which were generated in rats and generously supplied by Dr. Heinz W. Kunz, were utilized for Class I staining of rat cells (21). Anti-H-2^b monoclonal antibody (B22-249, R1, Serotec, Bioproducts for Science, Indianapolis, IN) was initially utilized for staining B10 cells. However, because of cross reactivity on F344 cells, this was substituted with 28-8-6S (IgG2a) obtained from ATCC and characterized in our laboratory. Further subset-specific markers in the rat including CD4 (W3/25) (Serotec) (22); CD8 (OX8) (Serotec) (22); monocytes, OX41 (Serotec) (23); Thy 1.1 (Serotec) (24); the α/β T-cell receptor (TCR), R73 (Serotec) (25); CD5 (Serotec) (26); CD3 (27) (generously supplied by Dr. Yoshiyuki Hasmimoto), and anti-rat NK (3.23; generously supplied by Dr. John Hiserodt) (28) were utilized for staining and lineage-specific analyses. All subset-specific monoclonal antibodies were species-specific and non-cross reactive on the irrelevant species. Data were displayed as cell frequency histograms in which log fluorescence intensity was displayed on the horizontal (x) axis and relative cell number on the vertical (y) axis. The percentage of cells considered positive after staining with the relevant monoclonal antibody was calculated using a cut-off for positivity determined from the control fluorescence profiles of negative and positive control populations (B10 mouse and F344 rat).

Monoclonal Antibodies for Staining. The following monoclonal antibodies were used FITC-conjugated: CD4, CD8, CD5, R7.3, Thy 1.1, and 3.2.3. FITC-conjugated Leu 4 (Becton Dickinson and Company, Mountain View, California) was used as an irrelevant control for background staining. CD3 is an IgM isotype and anti-IgM-FITC was used as a sandwich stain.

Skin Grafting. Skin grafting was performed by a modification of the method of Billingham and Medawar as previously described (14, 21). Full thickness skin grafts were harvested from the tails of F344 (Rt1A¹), and Wistar-Furth (Rt1A^u) rats and C57BL/10Sn (H-2^b) and C3H (H-2^k) mice. Mice were anesthetized with tribromoethanol intraperitoneally (29) and full thickness graft beds prepared surgically in the lateral thoracic wall. Care was taken to preserve the panniculus carnosus. The grafts were covered by a double layer of vaseline gauze and a plaster cast to prevent shearing. If more than one skin graft was placed on an animal, each defect for graft placement was separated by a 3 mm skin bridge. Casts were removed on the eighth day. Grafts were scored daily for percent rejection, and rejection was considered complete when no re-

sidual viable graft could be seen. Chronic rejection was the time point at which erythema and induration appeared in the grafts. Graft survivals were calculated by the life-table method (30) and the median survival time (MST) was derived from the time point at which 50% of grafts were surviving.

Red Blood Cell and Platelet Phenotyping: Glucose Phosphate Isomerase-1 Assay (GPI-1). Typing of red blood cell phenotype was performed by GPI-1 assay (31). The precipitation pattern for F344 rat or WF rat and B10 mouse were performed as controls and determined to be totally disparate. Briefly, 8 μ l of RBC were lysed in 400 μ l of distilled water, and electrophoresis was performed on Titan III cellular acetate strips with tris HCl 20 mM glycine 200 mM buffer (pH 8.7) (200V for 1 hour). Application was 2 cm from anode. After the run, the strips were covered with a 1% agarose gel containing tris-HCl 100 mM Ph 8.0, NADP 300 μ M, glucose-6-phosphate dehydrogenase 0.5 U/ml, fructose-6-phosphate 50 mM, MTT 500 μ M, phenazine methosulphate 200 μ M. As precipitation occurred with the formation of formazan salt, the bands became visible (blue). The gel was removed, the reaction was arrested by immersing the strips in 5% acetic acid, and the bands were scanned with a Quick-Scan scanner. Percentages were determined by comparison with the positive control. Values for each individual chimera were normalized to 100%. After isolation, platelets were typed in a similar fashion. B10 mouse and F344 rat were demonstrated have different precipitation patterns. This technique is able to reliably detect as low as 2% of contaminating rat RBC artificially titrated into RBC from normal B10 mice in our hands.

Platelet Isolation. Whole blood (0.9 ml) was drawn by cardiac puncture into a syringe containing 0.1 ml Na₃ Citrate, 130 mM, gently mixed and transferred into a microcentrifuge vial. The blood was spun for four seconds at the maximal setting (14,000 rpm) of an Eppendorf microcentrifuge (Beckman #5415). This setting was chosen through an optimization strategy in which force and times were varied as a function of retrieved platelet number. This duration included the acceleration phase, which is incomplete when power is curtailed at the four second mark. After this, the samples were allowed to slow to a halt without braking. Platelet-rich plasma was then carefully aspirated with a disposable polyethylene pipette, avoiding any disturbance of the buffy coat. Triplicate platelet counts were obtained using a Coulter Model ZB1 counter (Hialeah, FL), and the average (variation 5%) calculated. Platelets were then processed as described for the glucose phosphate isomerase-1 assay.

Results

Survival of Fully Xenogenic Chimeras (Non-TCD F344 Rat \rightarrow B10 Mouse). To characterize the ability to achieve successful bone marrow rescue of lethally irradiated normal B10 recipient mice with F344 rat bone marrow, fully xenogenic chimeras (non-TCD F344 rat \rightarrow B10 mouse) were prepared. Survival of animals was excellent; 100% at 50 days and 80% at 180 days (Fig. 1). Animals appeared healthy, without apparent wasting, anemia, dermatitis or diarrhea associated with graft versus host disease (GVHD). Survival was similar to that for syngeneically reconstituted mice (B10 \rightarrow B10) and mixed xenogeneically reconstituted mice which received a mixture of B10 mouse plus untreated rat bone marrow (B10 + non-TCD F344 \rightarrow B10) (Fig. 1). Unreconstituted radiation controls died within ten days.

Flow Cytometry Typing of Fully Xenogenic Chimeras: Evidence for Xenogenic Lymphoid Chimerism. All recipient mice

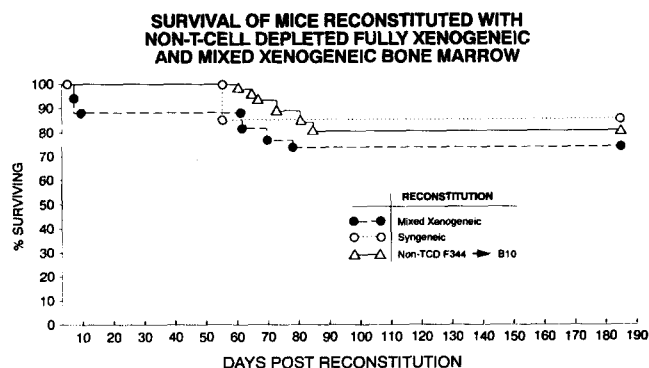


Figure 1. Life-table survival of fully xenogenic chimeras (non-TCD F344 rat \rightarrow B10 mouse) (N = 36). Syngeneically reconstituted mice (B10 \rightarrow B10) (N = 10) and mixed xenogenic chimeras (B10 + non-TCD F344 \rightarrow B10) (N = 10) which received a mixture of syngeneic B10 plus untreated F344 rat bone marrow were prepared as controls. Minimum follow-up was 77 d. Experimental animals prepared for lineage-specific analysis by flow cytometry were not included in survival analyses since sacrifice of the animals was required.

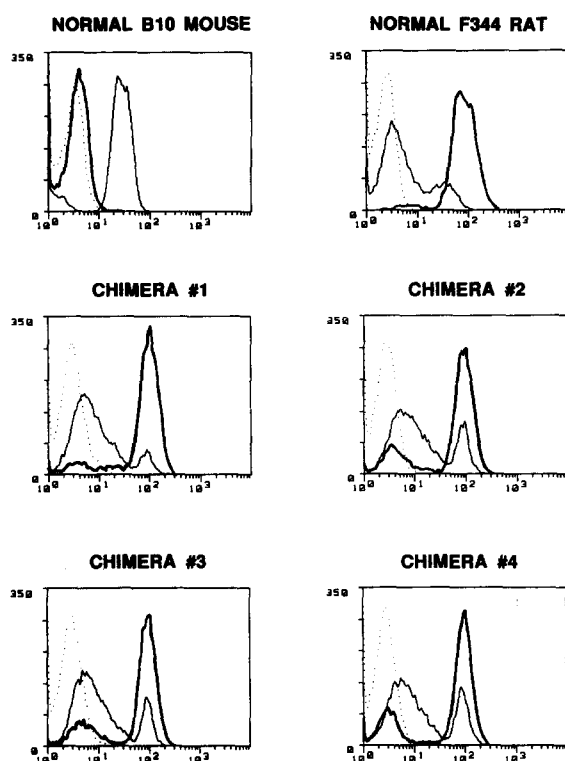
tested (N = 42) exhibited xenogenic rat chimerism when typed for rat peripheral blood lymphocytes (PBL) from one month to 7 mo post-reconstitution. Table 1 shows typing data from representative animals 4 months post reconstitution. Chimerism was stable over time with from 82 to 97% of total peripheral lymphoid cells of rat derivation, demonstrating that engraftment of xenogenic rat lymphoid elements could be successfully achieved in B10 mouse recipients. The sensitivity of this assay was determined by titration ex-

Table 1. Flow Cytometry Typing of PBL From Fully Xenogenic Chimeras*

| Group | Animal | Reconstitution | Rat (%) |
|-------|-------------|--------------------------------|---------|
| 1 | 1 | Non-TCD F344 \rightarrow B10 | 76.1 |
| | 2 | | 80.9 |
| | 3 | | 83.4 |
| | 4 | | 77.8 |
| | 5 | | 91.0 |
| | 6 | | 84.5 |
| 2 | 7 | Non-TCD WF \rightarrow B10 | 88.0 |
| | 8 | | 79.4 |
| | 9 | | 92.0 |
| | 10 | | 86.5 |
| | 11 | | 81.3 |
| | Normal B10 | | 2.5 |
| | Normal F344 | | 98.2 |
| | Normal WF | | 99.0 |

Typing was performed by flow cytometry using anti-H-2^b (28-8-6S) and anti-F344 or anti-WF mAbs. Results are from one representative experiment for animals 4 mo following reconstitution.

TYPING OF CHIMERAS



FLUORESCENCE INTENSITY

Figure 2. Representative flow cytometry histogram PBL typing of normal B10, normal F344, and 4 representative fully xenogeneic chimeras 4 mo following reconstitution. Anti-B10 (H-2^b) and anti-F344-biotin mAbs were used for one color staining. No crossreactivity occurred with the anti-F344 antibody on normal B10 lymphoid cells. However, there was moderate cross-reactivity of staining with the anti-B10 mAb (B22-249) on normal F344 lymphoid cells. (· · ·) isotype-specific fluorescence control (IgG2a or FITC-conjugated Leu 4); (---) anti-B10 mouse; (—) anti-F344.

periments in which 0.5% of contaminating rat lymphocytes could be reliably detected when added to normal B10 lymphocyte preparations. A representative flow cytometry typing experiment is shown in Figure 2. Similar results were obtained in a Wistar Furth (WF) rat → B10 mouse combination (Table 1; Group 2) and using B10.BR (H-2^k) or C3H (H-2^k) as recipients of either WF or F344 bone marrow (data not shown).

Specificity of Tolerance to Rat and Mouse in Fully Xenogeneic Chimeras (non-TCD F344 → B10). To determine the presence and specificity of transplantation tolerance induced and to assess competence to recognize and reject third party mouse and rat grafts, skin grafts from donor (F344; Rt1.A¹) and third party (WF; Rt1.Aⁿ) rat and third party mouse (C3H; H-2^k) strains were placed on the fully xenogeneic chimeras (Fig. 3). Survival of donor-specific skin grafts was specifically

prolonged (MST > 130 days), while MHC-disparate third party mouse and rat grafts were promptly rejected (MST = 10 days and 20 days respectively) with a time course similar to normal B10 mice. These experiments demonstrate that the specificity of tolerance to rat was highly donor-strain specific and that competence to recognize MHC-disparate third party mouse was preserved in these animals.

Donor-type skin grafts initially appeared soft and healthy without evidence for rejection. However, skin grafts underwent a slow chronic rejection with development of progressive erythema and induration with onset from 35 to 45 days, and then followed a course consistent with prolonged chronic rejection. The appearance of the grafts was similar to that reported for *mixed xenogeneic chimeras* prepared with TCD rat bone marrow (B10 + TCD F344 → B10) previously described (11, 18), and we predict that eventual disappearance of the grafts may result. The course is one of protracted chronic skin graft rejection, and no change in level of rat lymphoid chimerism has been observed (data not shown).

Rat-Stem-Cell-Derived Lineage Analysis in Fully Xenogeneic Chimeras. Fully xenogeneic chimeras (non-TCD F344 → B10) were examined by flow cytometry for the presence of various rat-stem-cell-derived hematolymphopoietic lineages in splenic lymphoid tissue from 3 wk to 7 mo after reconstitution. The presence of all rat stem-cell-derived lineages would be strong evidence for successful engraftment and differentiation of pluripotent rat hematopoietic stem cells in the xenogeneic environment of a normal B10 mouse recipient. In all 15 animals examined, F344 rat B-lymphocytes, T-lymphocytes, NK cells, and monocytes, were demonstrated in the spleen of chimeras (Figure 4). The profile of staining was similar to that for a normal F344 rat (Figure 4). The proportion of B-lymphocytes ranged from 25-32% of total cells; CD5 positive cells from 33 to 41%; NK cells from 8.1 to 10.5%, and monocytes from 36.5 to 38.5% of total cells analyzed using the lymphoid/myeloid gate.

Erythrocyte and Platelet Phenotype in Fully Xenogeneic Chimeras. The presence of rat erythrocytes was determined in chimeras using the glucose phosphate isomerase-1 assay (GPI-1) (31), an enzyme for which we have demonstrated that the electrophoretic pattern of F344 rat and B10 mouse differ. In artificial titrations performed using isolated RBC, as low as 2% of contaminating rat RBC were detectable when added to normal B10 mouse RBC. The 10% error is similar to that experienced for trypan blue exclusion typing of lymphoid cells (14, 21). The proportion of red blood cells of rat origin present in the fully xenogeneic chimeras ranged from 85.7% to 100% of total RBC at 7 months following reconstitution (Table 2).

The same assay was performed for phenotyping of isolated platelets, which were nearly totally (>95%) of rat origin in all animals tested at 7 months following reconstitution (N = 12) (Table 3).

Flow Cytometry Analyses of Radioresistant B10 Mouse Splenic Lymphocytes. Radioresistant syngeneic B10 host cells were present in the splenic lymphoid tissue of chimeras at levels from 3 to 18% of total lymphoid cells (Fig. 2). Further subset-

SKIN GRAFT SURVIVAL: SPECIFICITY OF TOLERANCE

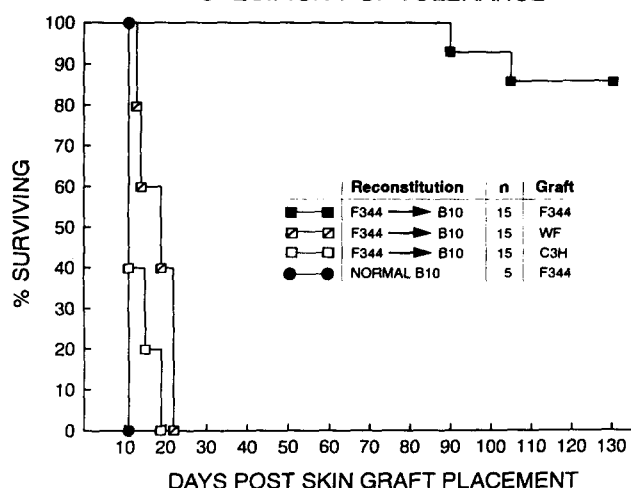


Figure 3. Survival of full thickness tail skin grafts placed six weeks post reconstitution (non-TCD F344 → B10). Each animal received a donor-specific F344, MHC-disparate third party rat (WF; Rt1.A^u), and MHC-disparate third party mouse graft (C3H; H-2^k). Survival was calculated by life table method (30), and grafts were followed for a minimum of 50 d. Grafts were scored daily for evidence of rejection, which was considered complete when no viable residual could be detected.

specific analyses demonstrated that the majority of these cells were Thy 1.2⁺ and CD4⁺. Murine CD8⁺ cells were also consistently detectable at low levels in the range of 1–5%, while B-cells, NK cells, and macrophages were not detected at the level of our 0.5% sensitivity. The nature of these ra-

Table 2. Typing of Red Blood Cell Phenotype*

| Animal | HCT [†] | Rat RBC | Mouse RBC |
|------------------------|------------------|---------|-----------|
| | | (%) | (%) |
| Chimera 1 [§] | 42 | 85.7 | 14.3 |
| Chimera 2 [§] | 41 | 90.1 | 9.9 |
| Chimera 3 [§] | 49 | 100 | 0 |
| Chimera 4 [§] | 32 | 100 | 0 |
| Chimera 5 [§] | 40 | 100 | 0 |
| Chimera 6 [§] | 43 | 100 | 0 |
| Normal B10 Mouse | 49 | 0 | 100 |
| Normal F344 Rat | 43 | 100 | 0 |

* One representative experiment for typing of red blood cell phenotype two months post-reconstitution by GPI-1 assay.

[†] Hematocrit.

[§] (F344 → B10).

dioreistant cells is uncertain, but they were clearly not sufficient to allow survival of the radiation controls.

Flow Cytometry Analysis of Rat-Derived T Cell Subsets and Markers Present in Splenic Lymphoid Tissue. The phenotype of rat-derived splenic lymphoid T-cell subsets in the fully xenogeneic chimeras was further characterized using species-specific monoclonal antibodies against rat Thy 1.1, the rat α/β -T-cell receptor, CD3 T-cell receptor complex, and rat T-cell subset markers including CD4, CD5, and CD8. Thy 1, a cell surface antigen present in large amounts on thymocytes

RAT LINEAGES PRESENT IN SPLEEN

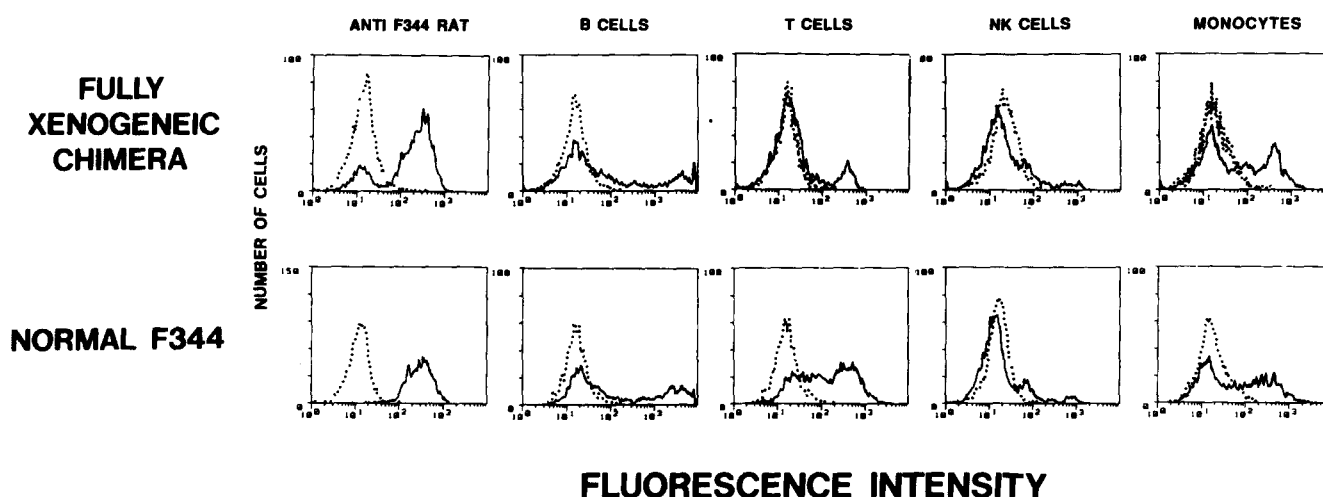


Figure 4. Sample flow cytometry analysis for the presence of rat-derived lymphoid, NK, and myeloid lineages obtained from splenic lymphoid tissue from a representative fully xenogeneic chimera 3 mo after reconstitution. Staining was performed in single color analysis with (A) anti-Class I F344 rat; (B) B-lymphocytes (OX6); (C) T lymphocytes (α/β -TCR); (D) Natural Killer Cells (3.23); and (E) monocytes (OX41). All antibodies were demonstrated to be non-cross-reactive on B10 splenic lymphoid cells. (· · ·) isotype-specific fluorescence control (Leu 4-FITC); (—) relevant mAb stain. These low cytometry analyses were all performed utilizing the lymphoid gate.

and brain tissue of rats and mice, exists in two allelic forms in the mouse (Thy 1.1 and Thy 1.2) and in only one allelic form in the rat (Thy 1.1) (25, 26). B10 mice express only Thy 1.2. In mice, Thy 1 is expressed on both mature T-lymphocytes and immature bone marrow cells (22, 23). In contrast, Thy 1.1 is a marker for only immature lymphocytes in the rat; it is present on up to 45% of immature bone marrow cells and on only a minority (<10%) of mature T cells in peripheral lymphoid tissue of the rat (25, 26). Thus, analysis of this marker in fully xenogeneic chimeras allowed us to assess whether rat-bone-marrow-derived T-cell precursors could undergo the normal maturational loss of Thy 1.1 in the murine stromal environment. All fully xenogeneic chimeras (N = 15) analyzed exhibited the normal F344 rat pattern of mature lymphocyte staining in peripheral lymphoid tissues: peripheral T-lymphocytes were rat-derived CD3⁺, CD4⁺, or CD8⁺, CD5⁺, α/β -T-cell receptor⁺, and Thy 1.1 negative or dull (Fig. 5). This staining pattern is characteristic of that for mature T-lymphocytes in the normal rat (Fig. 5).

Phenotype of Rat-Derived T Lymphocytes in Thymus of Fully Xenogeneic Chimeras. To determine whether rat T cells from the chimeras were derived from bone marrow precursors or from contaminating mature T lymphocytes transferred with the untreated rat bone marrow inoculum, we examined the thymocytes from chimeras specifically for T cell receptors normally acquired in the thymus. It has been demonstrated in mice that T cell maturation and receptor expression are developmentally regulated in the thymus during interaction with host thymic stroma. Acquisition of the T cell receptor complex occurs simultaneously with differentiation from double-positive (CD4⁺, CD8⁺) to single-positive CD4⁺ or CD8⁺ T-cells (32–34). An analogous maturation process is believed to occur in the rat (25, 27). Rat-specific T lymphocyte staining patterns in the thymic lymphoid tissues of fully xenogeneic

Table 3. Phenotype of Platelets in Xenogeneic Chimeras* (WF → B10 or F344 → B10)

| Group | Animal | Reconstitution | Rat platelets (%) | Rat lymphoid cells (%) |
|-------|------------------|----------------|-------------------|------------------------|
| 1 | 1 | F344 → B10 | 98.8 | 90.9 |
| | 2 | | 95 | 85.3 |
| | 3 | | 100 | 77.3 |
| | 4 | | 100 | 85.1 |
| | 5 | | 100 | 75.8 |
| | 6 | | 100 | 79.9 |
| | 7 | | 100 | NT† |
| | 8 | | 100 | NT |
| | 9 | | 99.0 | NT |
| 2 | 1 | WF → B10 | 99.2 | 86.7 |
| 3 | Normal B10 mouse | | 0 | |
| | Normal WF rat | | 100 | |
| | Normal F344 rat | | 100 | |

* Phenotype of platelets was determined by GPI-Isomerase assay, an enzyme for which B10 mouse and WF rat and F344 rat differ.

This group of animals was typed two months post reconstitution. Lymphoid typing was performed by flow cytometry 7 d after platelet typing.

† NT = Not tested.

chimeras were analyzed by flow cytometry analysis for the pattern of Thy 1.1, α/β -TCR, CD3, CD4, CD5, and CD8 staining (Fig. 6). In all (N = 15) animals examined, a staining

RAT T CELL SUBSETS IN SPLEEN

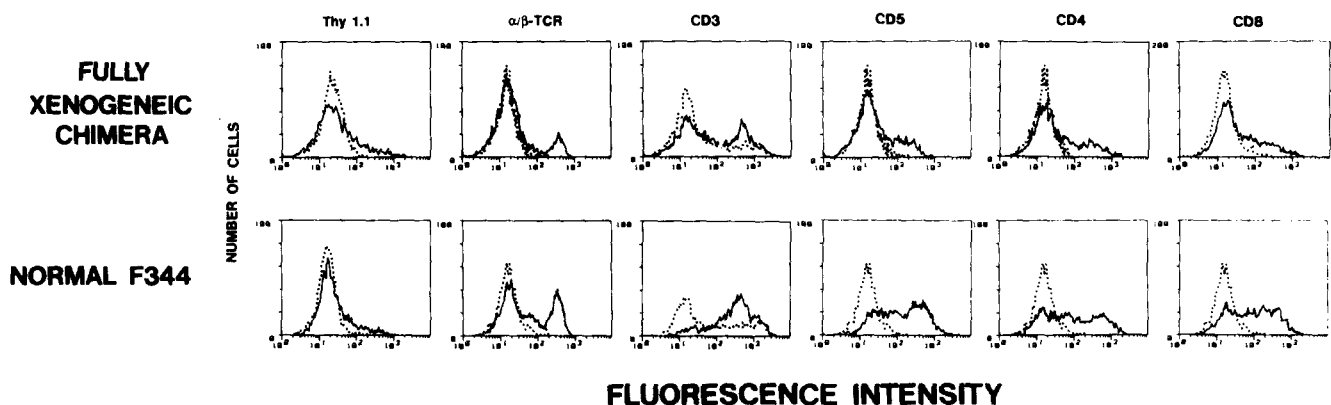


Figure 5. Representative flow cytometry analysis of T cell subsets present in the spleen of a fully xenogeneic chimera and control F344 rat 6 mo after reconstitution. Single color staining was performed using anti-rat Thy 1.1, anti- α/β TCR (R73), anti-CD3, anti-CD5, anti-CD4, and anti-CD8. R73 is a mouse anti-rat mAb which has been characterized to detect a rat pan-T-cell surface antigen compatible with the predicted properties of the α/β -TCR on mature and immature T cells (24). Anti-CD3 binds to the rat T cell receptor complex (25). CD5 binds to T cells and some activated B cells. (· · ·) Leu 4-FITC control for all antibodies except CD3 which requires an IgM sandwich; (—) relevant mAb stain.

RAT T CELL LINEAGES IN THYMUS

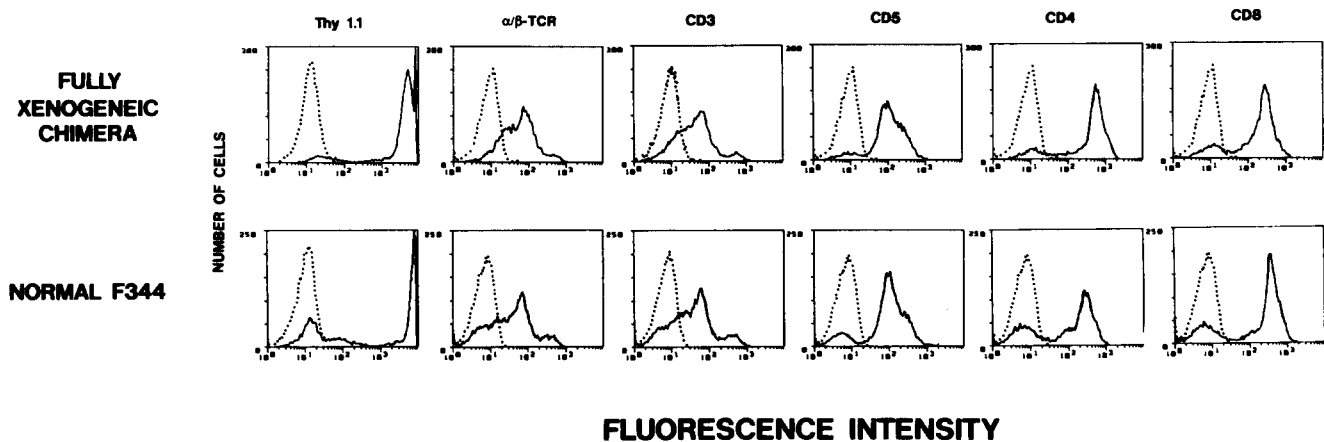


Figure 6. Representative FACS analysis of T cell CD subsets present in the thymus of a fully xenogeneic chimera and control F344 rat. Staining was performed using anti-rat Thy 1.1, anti α/β -TCR, anti-CD3, anti-CD5, anti-CD4, and anti-CD8 mAb as in previous experiments. (· · ·) Leu 4-FITC control; (—) relevant mAb stain. Animal is the same as that in Fig. 5.

pattern identical to that for normal thymic maturation of rat T-cells was present (Figure 6). A large proportion of Thy 1.1 positive bright rat T-cells was present in the thymus of both the normal rat and all fully xenogeneic chimeras, compatible with an immature staining pattern. CD3 and α/β -TCR staining were dull for both the chimeras and the normal thymus, indicating a predominance of immature rat T lymphocytes traversing the thymus of chimeras and the normal rat thymus. CD4 and CD8 staining patterns were also nearly identical for the normal rats and the fully xenogeneic chimeras. Most importantly, a "mature rat" fluorescence pattern for staining with anti-CD3 was present in the peripheral splenic lymphoid compartment of the chimeras (Figure 5 and 6), further supporting the concept that T-cell maturation was occurring with acquisition of the CD3-TCR complex in the xenogeneic thymic stromal environment of the chimeras.

Most rat-derived T cells in the thymus were phenotypically immature in staining pattern, expressing double-positivity with both CD4 and CD8-positive antigenic determinants when analyzed by flow cytometry (Fig. 7 A). As expected, double negative ($CD4^-$ and $CD8^-$) and single positive ($CD4^+$ or $CD8^+$) cells were present in low proportions. In contrast, the majority of rat-derived splenic lymphoid cells were single-positive CD8 or CD4 (Fig. 7 B). To determine that the rat antigenic determinants expressed on the splenic and thymic cells from chimeras were not picked up and expressed by mouse cells, double staining using anti-rat and anti-mouse T cell markers was performed (Fig. 8). Although a small percentage of mouse-derived CD4 and CD8 positive cells were present, the Thy 1.2 positive mouse cells were not positive for rat CD4 or CD8, suggesting that true engraftment of the rat stem cell with rat-derived T cell production and maturation was present in the fully xenogeneic chimeras.

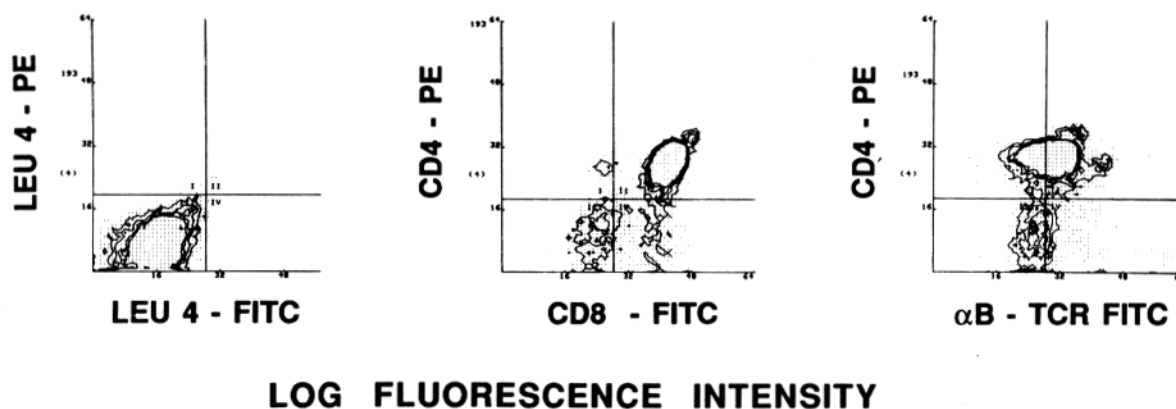
Discussion

Cross-species transplantation has been suggested as one potential solution for the current shortage of solid organs for transplantation which exists in the clinical setting. However, conventional nonspecific immunosuppressive agents have not been effective in controlling rejection in even closely matched species combinations (5). The mechanisms responsible for graft rejection in xenogeneic transplantation have not been well characterized, and may, in fact, differ from that for allogeneic disparities (35). It was recently reported that xenogeneic skin graft rejection is mediated primarily by CD4-positive lymphocytes (35). Before xenogeneic transplantation can be applied clinically, a better understanding of the mechanisms which mediate xenograft rejection and acceptance is required.

The preparation of allogeneic chimeras using numerous approaches has been demonstrated to induce donor-specific transplantation tolerance (10–19), and it has been suggested that the induction of xenogeneic chimerism through cross-species bone marrow transplantation may overcome the vigorous rejection which occurs when solid organs are transplanted into disparate species. Successful engraftment of rat bone marrow into mouse recipients was reported in the 1950's (6–9). Production of rat-derived leukocytes and RBC was demonstrated (6–8), as well as acceptance of donor skin grafts (9). However, these studies were limited by poor survival and the limited technology for typing of recipients. We now report the stable long-term engraftment of rat bone marrow into irradiated mouse recipients. Multi-lineage production of bone marrow-derived cells, including RBC, NK, lymphoid, myeloid cells, and platelets, persisted up to 7 months following reconstitution, suggesting engraftment of the rat stem cell or self-renewing bone marrow progenitor cells. Successful

A

2 COLOR FACS ANALYSIS: T CELL MARKERS IN THYMUS



B

2 COLOR FACS ANALYSIS: T CELL MARKERS IN SPLEEN

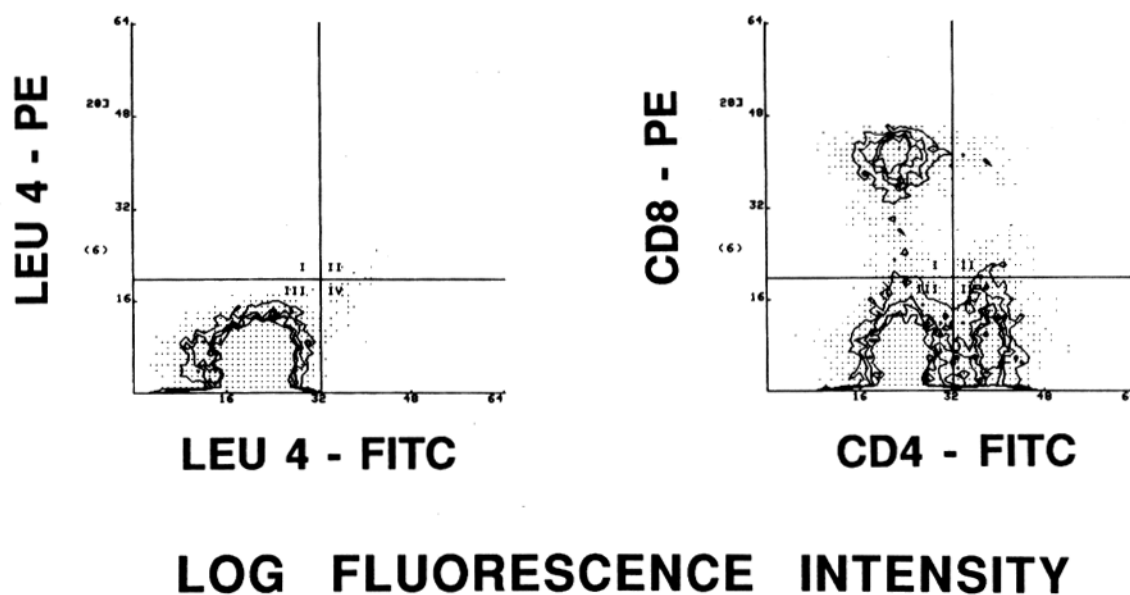


Figure 7. (A) Two color flow cytometry typing of CD4 and CD8 rat staining patterns for rat-derived thymic lymphoid cells at 7 mo following fully xenogeneic reconstitution. Anti-rat CD8-FITC and CD4-PE were utilized for staining. Leu 4 FITC and Leu-4-PE were the irrelevant controls. $\alpha\beta$ TCR rat versus rat CD4 staining is also shown. (B) Two color flow cytometry analysis of CD4 and CD8 staining patterns for rat-derived splenic lymphoid cells in same animal.

engraftment of rat bone marrow cells using a number of MHC and minor-disparate strain combinations suggests that this may be a generalizable model for closely related species combinations. This model can now be exploited to study mecha-

nisms responsible for tolerance induction across a species barrier.

The specificity of tolerance induced by fully xenogeneic reconstitution (rat \rightarrow mouse) was highly specific for both

2 COLOR ANALYSIS OF RAT VS. MOUSE DETERMINANTS IN SPLEEN

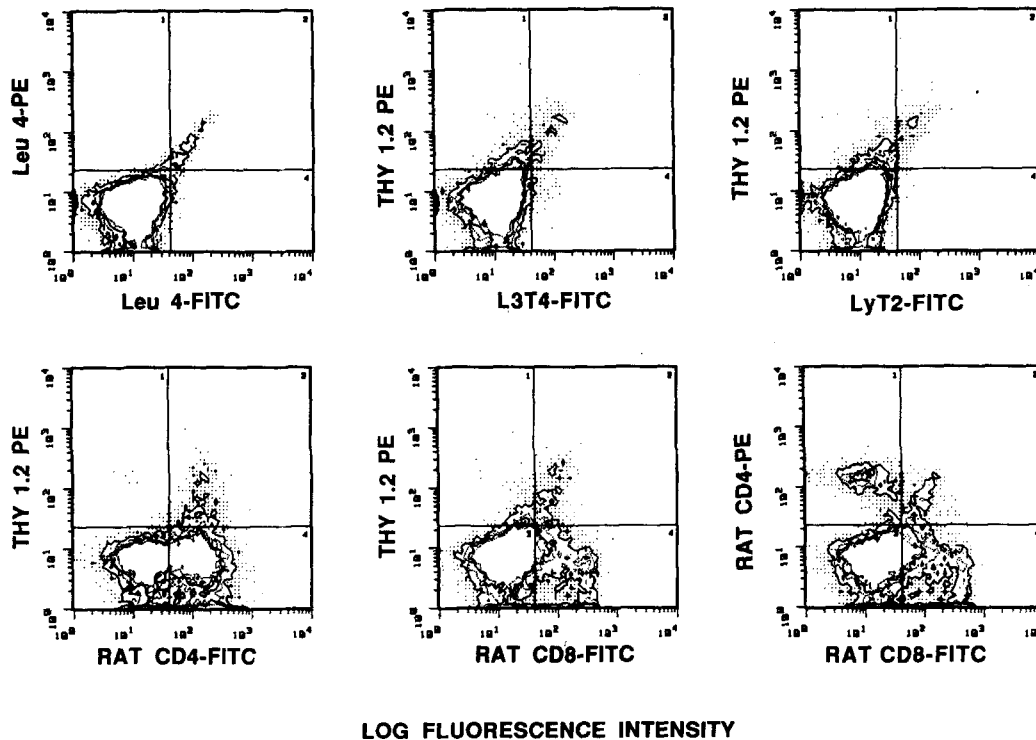


Figure 8. Two color flow cytometry analysis of splenic lymphoid cells from fully xenogeneic chimeras was performed to determine whether the rat T cell markers present were actually picked up by radioresistant B10 mouse cells. Anti-mouse T cell markers included anti-mouse T cell (Thy 1.2), anti-mouse CD4 (L3T4-FITC), and anti-mouse CD8 (Lyt2-PE). Two-color staining was performed versus anti-rat CD4 and anti-rat CD8-FITC. Leu 4-PE and Leu 4 FITC staining were performed as controls.

MHC-disparate third party rat and mouse. (Rat → mouse) xenogeneic chimeras did not see rat as generic rat or mouse as generic mouse, but each as MHC-specific strain since the chimeras were competent to recognize and reject MHC-disparate third party rat and mouse skin grafts. Whether this effect was mediated by rat lymphocytes, mouse lymphocytes, or both is currently under investigation in our laboratory. Even after the onset of chronic rejection in donor skin grafts, chimerism persisted unchanged. We speculate that this chronic graft rejection is due to antigens present on skin, but not the tolerizing lymphoid cells, and is similar to the skin-specific antigens characterized in an allogeneic model by Boyse and Old (37) and Steinmuller (38). Similar findings have been observed in mixed xenogeneic chimeras (TCD B10 + non-TCD F344 → B10) (manuscript submitted). Studies are in progress to evaluate the effector and target cells responsible for this phenomenon.

We previously reported that stable engraftment of rat bone marrow could not be achieved in murine recipients when the marrow inoculum was depleted of T-lymphocytes (15, 20). In contrast, excellent survival resulted in the present studies when irradiated B10 recipients were reconstituted with un-

treated rat bone marrow. The excellent survival of fully xenogeneic chimeras (non-TCD F344 rat → mouse) prepared with untreated rat bone marrow is suggestive that cells in the rat bone marrow inoculum which express T-cell markers exerted a facilitatory effect on engraftment of rat bone marrow in murine recipients across a species barrier. Numerous reports in the literature have demonstrated that T-cells play a critical role in facilitation of engraftment of allogeneic bone marrow in both the clinical setting and in animal models (37). The present data suggest that the presence of cells which express T-cell markers in the xenogeneic rat bone marrow inoculum may similarly facilitate engraftment of rat bone marrow in mouse recipients across a species barrier. Rabbit-anti-rat brain sera (anti- θ) was used in the past to perform T-cell depletions of rat bone marrow (39, 40). However, since Thy 1 is present only on immature rat T-cells, this agent may not be effective at T-cell depletion. The influence of T-cell depletion of rat bone marrow on engraftment across a species barrier is difficult to address because of the lack of complement-fixing anti-rat-T-cell monoclonal antibodies. Cellular depletions utilizing immunomagnetic beads allows at best a 1 log reduction, which is insufficient compared with the 2 to 3 log reductions which

result with monoclonal antibody plus complement treatment of splenic lymphoid cells or bone marrow (personal observation). Therefore, we can only speculate that engraftment of rat stem cells may be facilitated by the presence of cells which express the theta (θ) antigen.

The presence of rat-T-lymphocytes with an immature staining pattern in the thymus and mature phenotype in the periphery was strongly suggestive that rat T-lymphocytes were not only capable of maturation but also the gene rearrangement required to express the T-cell receptor complex in a xenogeneic stromal environment of the chimeras. The immature rat staining pattern observed in the thymus of the fully xenogeneic chimeras strongly suggests that the α/β -TCR-positive T-lymphocytes detected in the peripheral lymphoid tissues were not derived from mature T-lymphocytes contaminating the untreated rat bone marrow at the time of transplantation, but instead from bone marrow-derived precursors which interacted with the thymic stroma to differentiate in a "normal rat" pattern. Maturation of immature rat T-lymphocytes in the thymus of xenogeneic chimeras followed a pattern similar to normal rat, with a high proportion of immature Thy 1.1 bright double-positive ($CD4^+$, $CD8^+$) T-cells in the thymus but not in the periphery, and a fairly high percentage of $CD3$ and α/β -TCR dull T-lymphocytes in the thymus compared with the bright, separate peaks for $CD3$ and α/β TCR plus $CD4$ or $CD8$ single-positive staining in the peripheral lymphoid tissue.

The absence of obvious graft versus host disease in fully xenogeneic chimeras prepared with untreated rat bone marrow in our clean facility raises the question of whether GVH disease can be induced across a species barrier. The contribution of GVH-reactivity versus immunoincompetence to the "wasting disease" and mortality observed in fully allogeneic chimeras ($A \rightarrow B$) prepared using allogeneic strain disparities has been debated. Zinkernagel (41) and Rayfield and Brent

(42) have demonstrated that immunoincompetence independent of GVH disease can result in the wasting appearance and poor survival of fully allogeneic chimeras. This effect is postulated to be secondary to a failure of appropriate interaction between donor-derived antigen presenting cells and lymphocytes restricted to see antigen in the context of the syngeneic MHC of the host thymus in which they matured (43-45). Previous attempts at transplantation of rat bone marrow into mouse recipients in the 1950's in a conventional animal facility were limited by poor survivals attributed to GVH disease (6-9). However, since there is no objective assay for GVH disease, this could have been due instead to immunoincompetence similar to that observed in fully allogeneic chimeras. Studies are in progress to characterize the specificities of lymphocyte interactions and self-restriction in our current system.

In summary, transplantation of xenogeneic rat bone marrow into normal irradiated B10 mouse recipients resulted in a highly selective degree of donor-specific transplantation tolerance in which rat was not seen as generic rat but as specific strain of rat. Competence to recognize and respond to MHC-disparate third party mouse and rat was preserved. Stable multilineage production of rat-stem-cell-derived cellular products is suggestive that stem cell engraftment has occurred across a species barrier. This model may provide a method to characterize the conditions and cells responsible for the induction and maintenance of tolerance across a species barrier, as well as restriction specificity for lymphocyte interactions. In addition, the model may provide a method to study stem cell engraftment, species-specific growth factor and cytokine production, and maturation of hematolymphoid elements in a xenogeneic stromal environment. Studies are currently in progress to evaluate the role of clonal deletion in tolerance induction across xenogeneic disparities.

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