# IN VIVO AND IN VITRO CHARACTERIZATION OF SPECIFIC HYPOREACTIVITY TO SKIN XENOGRAFTS IN MIXED XENOGENEICALLY RECONSTITUTED MICE (B10 + F344 RAT → B10)

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Transplantation between genetically disparate individuals requires chemotherapeutic agents to suppress the rejection reaction. Although these agents are vital to the suppression of graft rejection, they have their own deleterious side effects caused by both nonspecific immunosuppression and drug toxicities. In addition, chronic rejection in varying degrees of severity often occurs despite the use of these agents.

Since Medawar and colleagues (1) reported the induction of specific transplantation tolerance in mice by neonatal injection of allogeneic bone marrow cells, the search for methods to induce specific tolerance in adult recipients has continued (2–5). We have recently shown (6) that reconstitution of lethally irradiated B10 mice with T cell–depleted syngeneic (B10) plus xenogeneic (F344 rat) bone marrow results in long-term survival of animals without apparent graft vs. host or wasting disease and leads to specific prolongation of donor-type, full-thickness tail skin grafts. Such animals promptly rejected third party allogeneic and xenogeneic skin grafts with a time course similar to that of unirradiated control animals.

Our hypothesis for explaining these preliminary results was that syngeneic components offered immunocompetence, while xenogeneic elements led to hyporeactivity across a species barrier. However, neither the extent or specificity of this phenomenon as assessed by in vivo and in vitro parameters nor the nature of T cell responsiveness had been characterized in these animals. In this paper, we present data detailing the extent and specificity of hyporeactivity achieved across a species barrier. Mixed reconstituted animals are shown to exhibit specific hyporeactivity to donor antigens as assessed by skin grafting and by in vitro assays, including mixed lymphocyte culture proliferative and cellular cytotoxicity. These animals were immunocompetent, as evidenced by rejection of third party mouse and rat skin grafts and development of anti-sheep red blood cell plaque-forming responses equivalent to those of syngeneic controls. This new approach offers a model for investigation of mechanisms of self-recognition and induction and maintenance of transplantation tolerance across species barriers.

## Materials and Methods

*Animals.* 6–8-wk-old male C57BL/10Sn (B10), B10.BR, and B10.D2 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. 4–8-wk-old Fischer 344 (F344) and Wistar-Furth (WF) male rats were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, MA. Animals were housed in a specific pathogen-free facility at the National Institutes of Health (NIH), Bethesda, MD.

*Mixed Xenogeneic Reconstitutions: (B10 + F344 → B10).* Mixed xenogeneically reconstituted animals were prepared by a modification of the method previously described for preparation of allogeneic chimeras (7). Briefly, inbred C57BL/10Sn male recipients were lethally irradiated with a single dose of 950 rad from a cesium source. Using sterile technique, the bone marrow was flushed with Media 199 (Gibco Laboratories, Grand Island, NY) containing 50 μg/ml gentamicin (minimum essential medium [MEM])[1] from the femurs and tibias of C57BL/10Sn male mice and F344 male donors with a 23-gauge needle. The marrow was mechanically resuspended in MEM by gentle aspiration through a 19-gauge needle and the suspension was sterilely filtered through nylon mesh. The cells were then pelleted at 1,200 rpm for 10 min, resuspended, and counted. Marrow cells were T cell depleted by treatment with a 1:60 dilution in MEM of rabbit anti-mouse brain (RαMB) ($1 \times 10^8$ cells/ml of RαMB) at 4°C for 30 min (7). The cells were subsequently washed and resuspended in sterile filtered guinea pig complement (Gibco Laboratories) at a 1:3 dilution in MEM ($1 \times 10^8$ cells/ml) at 37°C for 30 min. Cells were then washed twice and resuspended in MEM at the appropriate concentration to allow injection of 1 ml per animal. Recipient animals were reconstituted within 4–6 h of lethal irradiation via the lateral tail veins using a 27-gauge needle. Mixed xenogeneically reconstituted animals received $5 \times 10^6$ T cell–depleted bone marrow cells and $4.0 \times 10^7$ F344 T cell–depleted rat bone marrow cells unless otherwise specified. Such animals will be referred to as mixed xenogeneically reconstituted mice or B10 + F344 → B10. Fully xenogeneically reconstituted animals received $4.0 \times 10^7$ T cell–depleted F344 rat bone marrow cells. Radiation controls were prepared simultaneously to confirm adequacy of the radiation.

*Fully Allogeneic Reconstitutions: (B10.D2 → B10).* Fully allogeneic chimeras were prepared using a technique identical to the mixed xenogeneically reconstituted animals. B10 recipients were reconstituted with $1.5 \times 10^7$ T cell–depleted B10.D2 bone marrow cells.

*Assays for Chimerism.* Chimerism of peripheral blood lymphocytes was assayed using a trypan blue exclusion microcytotoxicity test to determine the percentage of lymphocytes bearing donor or host-type H-2 or Rt1 surface markers as previously described (8). Briefly, peripheral blood was collected into heparinized plastic tubes. After thorough mixing, the suspension was diluted with 100 μl of saline and layered over a cold Ficoll-Hypaque gradient (Bionetics Laboratory Products, Kensington, MD). Following centrifugation for 30 s in a microfuge (Beckman Instruments, Inc., Fullerton, CA), the lymphocyte layer was aspirated from the saline-Ficoll interface and washed with MEM plus 0.1% gelatin. Cells were then used in the standard trypan blue microcytotoxicity assay (8).

Chimerism of spleen, lymph node, peripheral blood, bone marrow, and thymus was further assayed using a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) as previously described (9). Mouse anti-rat antiserum and a fluorescein isothiocyanate–conjugated goat $F(ab')_2$ anti-mouse IgG preparation (FITC-GAMIgG) (Cappel Laboratories, Cochranville, PA) were used for staining.

*Skin Grafting.* Skin grafting was performed by a modification of the method of Billingham (10). Full-thickness skin grafts were harvested from the tails of F344 ($Rt1^a$) and WF ($Rt1^u$) rats and C57BL/10Sn ($H\text{-}2^b$) and B10.D2 ($H\text{-}2^d$) mice. Mice were anesthetized with tribromoethanol administered intraperitoneally (11) and full-thickness graft beds were created surgically in the lateral thoracic wall with care taken to preserve the panniculus carnosum. The grafts were covered by a double layer of vaseline gauze and a

[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; Con A, concanavalin A; FACS, fluorescence-activated cell sorter; FITC-GAMIgG, fluorescein isothiocyanate-conjugated goat anti-mouse IgG; FMF, flow microfluorometry; MEM, minimum essential medium; MLR, mixed lymphocyte reaction; PFC, plaque-forming cell; PHA, phytohemagglutinin; RαMB, rabbit anti-mouse brain; SRBC, sheep red blood cells.

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 or larger skin bridge. Casts were removed on the eighth day. Grafts were scored daily for percent rejection, and rejection was considered complete when no residual viable graft could be seen. Graft survivals were calculated by the life-table method and the median survival time (MST) was derived from the time point at which 50% of grafts were surviving.

*Splenectomy.* Partial splenectomy was performed under ether general anesthesia through a left subcostal oblique incision. The vessels supplying the segment of spleen to be harvested were crushed with a mosquito snap as they emanated from the splenic hilum and the distal segment of spleen was amputated with iris scissors. A two-layer closure with 3–0 silk was performed.

*Mixed Lymphocyte Reactions.* Mixed lymphocyte reactions (MLR) were performed as previously described (12). Briefly, murine and rat splenocytes were ACK-lysed (ammonium chloride potassium carbonate lysing buffer) (NIH Media Unit), washed, and reconstituted in EHAA (12) (NIH Media Unit) supplemented with 0.75% normal mouse serum, 0.09 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Either $2 \times 10^5$ or $4 \times 10^5$ responders were stimulated with $4 \times 10^5$ stimulators (2,000 rad) and varying numbers of co-cultured putative suppressors or control normal cells in a total of 200 or 250 μl of media. Cultures were incubated at 37°C in 5% $CO_2$ for 4 d, pulsed on the third day with 1 μCi [$^3$H]thymidine (New England Nuclear, Boston, MA), and harvested on the fourth day with an automated harvester (Mash II; Microbiological Associates, Bethesda, MD).

*Cell-mediated Lympholysis (CML).* Xenogeneic CML assays were performed using a modification of techniques described elsewhere (12–14). Briefly, RPMI 1640 medium (Gibco Laboratories) was supplemented as above, except that 10% fetal calf serum (Gibco Laboratories) was used in place of normal mouse serum. Additionally, rat lymph node target cells were cultured in media with 10% normal rat serum in lieu of fetal calf serum. $4 \times 10^6$ responders were co-cultured with $2 \times 10^6$ or $4 \times 10^6$ putative suppressors or control cells and $4 \times 10^5$ splenocyte stimulators (2,000 rad) in 2.5 ml of medium at 37°C for 5 d. Mouse target blasts were stimulated with concanavalin A (Con A) (Miles Yeda Research Products, Rehovot, Israel) for 2–3 d. Rat lymph node target cells were stimulated with phytohemagglutinin (PHA) (Gibco Laboratories) at a dilution of 1:400 for 3–4 d. After 5 d responders were harvested, counted, and resuspended at appropriate effector-to-target ratios with $1 \times 10^4$ $^{51}$Cr-labeled, 2–3-d Con A mouse splenocyte blasts or 3–4-d PHA rat lymph node blasts as targets. After 4.5 h, supernatants were harvested with the Titertek supernatant harvesting system and specific lysis was calculated as follows: specific lysis = (experimental release − spontaneous release)/(maximal HCl release − machine background) × 100. Spontaneous release was <25% of maximum release unless otherwise indicated.

*IgG1 and IgG2 Isotype Levels.* Levels of IgG1 and IgG2 against donor lymphoid elements in sera were assessed by a direct binding assay on the FACS using purified FITC-GAMIgG1 and IgG2 reagents (provided by Dr. Howard Dickler, National Institutes of Health) as previously described (9).

*Plaque-forming Cell (PFC) Assays.* Sheep red blood cell (SRBC) PFC assays were performed as previously described (15). Briefly, animals were immunized intravenously with 0.2 cc of a 1% SRBC suspension on day 0. Partial splenectomies were performed on day 5, splenic lymphoid cells were harvested as resuspended in Hanks' balanced salt solution with phenol red (M.A. Bioproducts, Walkersville, MD), and the standard plaque-forming assay was performed in triplicate at 1:10, 1:100, and 1:1,000 spleen cell equivalents to ensure the optimal number of plaques per slide. Values are reported as plaques per $10^6$ spleen cells. All experiments reported were read blind.

## Results

*Survival of Mice Reconstituted with Fully Xenogeneic or Mixed Xenogeneic Bone Marrow Inocula.* Because we were interested in reconstituting mice with xeno-

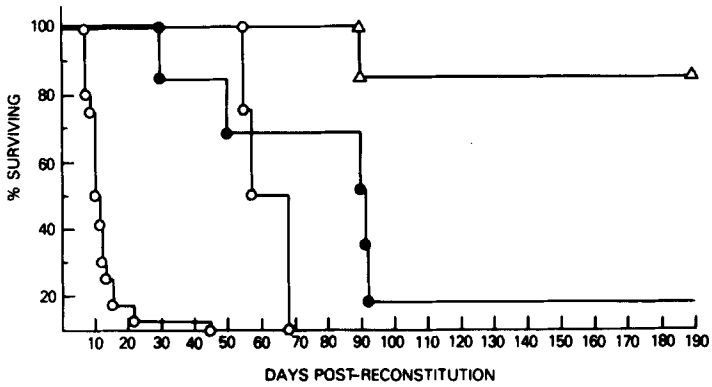


FIGURE 1. Survival of fully and mixed xenogeneically reconstituted mice as calculated by the life-table method. All bone marrow inocula were T cell depleted with RαMB plus complement. Fully xenogeneic: ○, $2.0 \times 10^7$, rat ($n = 24$); ●, $4.0 \times 10^7$ rat ($n = 6$); ⬡, $8.0 \times 10^7$ rat ($n = 4$). Mixed xenogeneic: △, $5 \times 10^6$ B10 + $4.0 \times 10^7$ rat ($n = 14$).

geneic bone marrow in an attempt to induce specific tolerance to subsequent xenografts, we first attempted fully xenogeneic marrow transplantation (F344 rat → B10). However, this approach was unsuccessful because survival of fully xenogeneically reconstituted animals was extremely poor, leading ultimately to death in all but one animal (Fig. 1). In contrast, B10 recipient mice reconstituted with a mixture of syngeneic and xenogeneic F344 rat bone marrow cells showed a long-term survival similar to B10 animals reconstituted with syngeneic bone marrow. Survival was 100% at 90 d and 86% at 190 d (Fig. 1). The mixed reconstituted animals all appeared healthy, showing none of the wasting and debilitation exhibited in control animals reconstituted simultaneously with identically treated rat bone marrow cells alone. All unreconstituted control animals died within 2 wk of irradiation.

*Titration of Syngeneic and Xenogeneic Bone Marrow Cells Required for Reconstitution.* Titrations were performed to determine the optimal number of syngeneic and xenogeneic T cell–depleted bone marrow cells required to achieve reproducible reconstitution and tolerance. Although $1 \times 10^6$ syngeneic bone marrow cells was sufficient for survival in 80% of animals, a dose of $5 \times 10^6$ cells was chosen since at this dose reconstitution was observed in 100% of animals (data not shown).

Optimal numbers of xenogeneic bone marrow cells required for induction of hyporeactivity was determined by skin xenograft survival in mixed xenogeneically reconstituted animals (Fig. 2). Mixed reconstitution of animals with $1.0 \times 10^7$ T cell–depleted F344 rat bone marrow cells or less led to prompt rejection of skin xenografts in 100% of animals, while animals reconstituted with greater than this number of cells exhibited prolongation of skin xenografts to varying degrees. In all animals, the grafts eventually underwent a chronic form of rejection with ultimate disappearance of the grafts in most animals. Although mixed reconstitution with $2.0 \times 10^7$ T cell–depleted F344 bone marrow cells led to specific skin xenograft prolongation in the majority of the animals, the onset of chronic rejection occurred more rapidly and was more severe than in

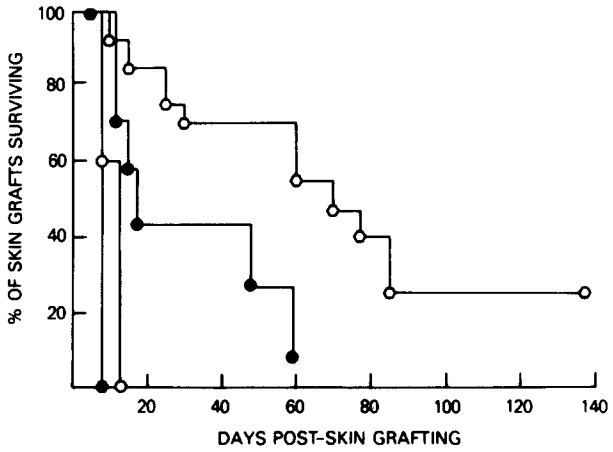


FIGURE 2. Dose-response effect of reconstitution with T cell–depleted F344 rat bone marrow on skin xenograft survival. Animals were reconstituted with $5 \times 10^6$ T cell–depleted syngeneic (B10) bone marrow cells plus varying numbers of T cell–depleted F344 rat bone marrow cells. ○, $1.0 \times 10^7$ R$\alpha$MB F344 (5); ●, $2.0 \times 10^7$ R$\alpha$MB F344 (7); ⬡, $4.0 \times 10^7$ R$\alpha$MB (12); ⬢, syngeneic (B10 → B10) (5).

mice reconstituted with greater numbers of rat bone marrow cells (Fig. 2). Reconstitution with 4.0, 6.0, or $8.0 \times 10^7$ identically treated F344 rat bone marrow cells resulted in prolongation of F344 xenografts with identical appearance (data not shown). All additional experiments reported in this study were performed with $5 \times 10^6$ syngeneic (B10) and $4.0 \times 10^7$ xenogeneic, F344, or WF T cell–depleted bone marrow cells.

*Typing of Mixed Xenogeneically Reconstituted Mice.* Typing of the mixed xenogeneically reconstituted animals by trypan blue exclusion microcytotoxicity assay revealed low numbers, if any, of detectable rat lymphoid elements in peripheral blood. Because the sensitivity of this assay is inadequate for accurate determination of such low percentages of lymphoid cells, further analysis of the animals was pursued by flow microfluorometry (FMF) using splenic lymphoid cells and MLR typing in which chimeric cells were used as stimulators of normal B10 responding cells.

Analyses performed by FMF (FACS II) revealed that 0.5% of F344 rat cells could be detected when mixed with B10 splenocytes and stained with mouse anti-rat sera. There was no cross-reactivity of the anti-H-$2^b$ monoclonal and mouse anti-rat sera used in staining on the inappropriate species. A fully xenogeneic chimera examined at 30 d post-reconstitution revealed 75% rat cells and 40% H-$2^b$-bearing cells present in splenic tissue (Fig. 3), which indicates that rat antigen could be detected in chimeric animals. Under these staining conditions, rat cells were detectable at very low levels in only 20% (5/25) of mixed xenogeneically reconstituted animals tested from 6 to 16 wk post-reconstitution. Fig. 3 illustrates one representative animal with a low level of rat cells detectable. Rat cells were not detectable in thymus, bone marrow, peripheral blood, and lymph node examined in similar fashion (data not shown) despite the persistence of an F344 skin graft for >50 d.

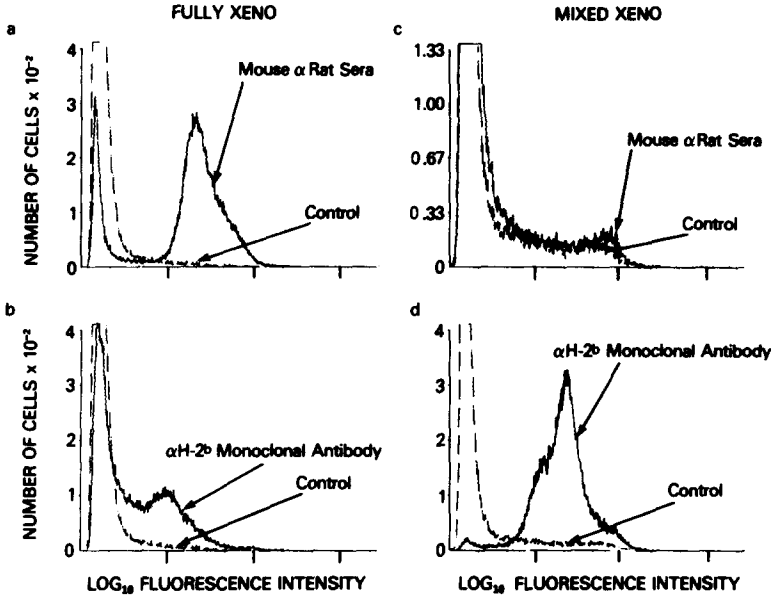


FIGURE 3. FACS analysis of fully (*a* and *b*) and mixed (*c* and *d*) xenogeneically reconstituted animals. Cells were stained with a monoclonal anti-H-$2^b$ antibody or mouse anti-rat sera, both demonstrated to have no cross-reactivity on inappropriate species cell type.

TABLE I
*Typing of Xenogeneically Reconstituted Animals by MLR*

| Responder* | Stimulator | cpm‡ ± SEM | Stimulation index§ |
|---|---|---|---|
| Chimera A | Chimera | 1,095 ± 246 | |
| Chimera | B10 | 1,183 ± 425 | |
| Normal B10 | B10 | 1,009 ± 27 | |
| Normal B10 | Chimera | 2,694 ± 251 | 2.7 |
| Chimera B | Chimera | 1,584 ± 202 | |
| Chimera | B10 | 1,397 ± 94 | |
| Normal B10 | B10 | 2,886 ± 920 | |
| Normal B10 | Chimera | 2,806 ± 295 | 1.0 |
| Chimera C | Chimera | 1,381 ± 42 | |
| Chimera | B10 | 947 ± 128 | |
| Normal B10 | B10 | 2,886 ± 920 | |
| Normal B10 | Chimera | 2,825 ± 1,065 | 1.0 |

* Analysis of spleens from three chimeras by MLR in attempt to detect donor-type F344 cells.
‡ Counts per minute of [$^3$H]thymidine incorporation.
§ Stimulation index compares ratio of normal B10 anti-chimera response to that of normal B10 anti-B10.

Further evaluation of mixed xenogeneically reconstituted animals was performed by MLR typing in which normal B10 responding cells were stimulated by irradiated chimeric cells. One representative experiment performed with spleen cells from three chimeras is shown in Table I. Most animals failed to

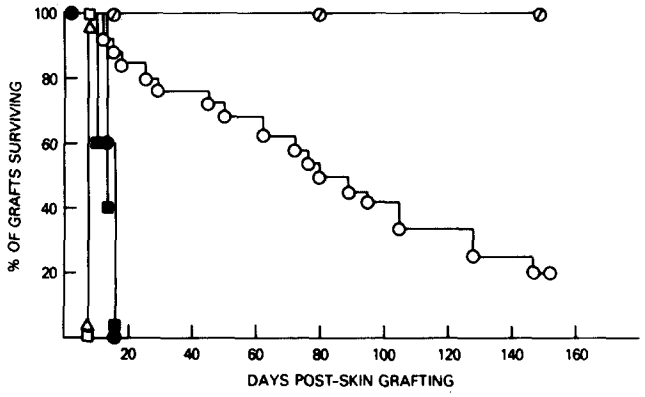


FIGURE 4. Skin xenograft survival in mixed xenogeneically reconstituted mice as calculated by the life-table method. Animals and skin grafts: ○, mixed xenogeneic, F344 (26); ●, mixed xenogeneic, B10.D2 (17); Ø, mixed xenogeneic, B10 (5); □, syngeneic, F344 (10); ■, syngeneic, B10.D2 (5); △, normal B10, F344 (10).

stimulate a proliferative response in normal B10 responding cells. However, some animals, such as chimera 1, did produce a response, which indicates the possible persistence of xenogeneic elements.

*Specific Hyporeactivity to Donor-Type Skin Grafts.* Mixed xenogeneically reconstituted animals demonstrated reproducible specific hyporeactivity to donor-type full-thickness tail skin grafts (MST = 80 d) (Fig. 4). However, rejection of third-party allografts (Fig. 4) and xenografts (Table II) followed a time course similar to that of syngeneically reconstituted animals (Fig. 4) and nonirradiated controls. The skin grafts varied in appearance and texture from soft to indurated, often with an appearance of prolonged chronic rejection. In the majority, the onset of the appearance of chronic rejection was from 20 to 90 d. However, in some animals, the grafts remained soft and uninflammed for up to 120 d.

To evaluate the degree of specificity of hyporeactivity to rat skin grafts, mice reconstituted with T cell–depleted syngeneic (B10) plus either T cell–depleted F344 or WF bone marrow were grafted with both F344 and WF tail skin simultaneously. All skin grafts were read blind for the first 33 d. Grafts were considered rejected when no viable residual could be detected. As shown in Table II, it was clear that hyporeactivity to donor-type skin was specific, since with few exceptions mice reconstituted with F344 bone marrow retained F344 skin grafts but specifically rejected WF skin grafts with a time course similar to syngeneically (B10 → B10) reconstituted animals. Conversely, mice reconstituted with B10 plus WF bone marrow were hyporeactive to WF skin but rejected F344 rat tail skin grafts with a time course similar to syngeneically reconstituted animals.

*Serologic Reactivity and Attempts to Detect Enhancement.* Sera obtained sequentially following skin grafting of mixed xenogeneically reconstituted animals were tested on F344 rat spleen target cells by complement-mediated cytotoxicity. Table III shows the cytotoxic titers and maximum percentage lysis of F344 target cells by sera from five representative animals. Included are data from one

TABLE II

*Skin Graft Specificity in Mixed Xenogenically Reconstituted Animals*

| Reconstitution | Animal number | Skin graft survival (days) | | | |
|---|---|---|---|---|---|
| | | F344 | MST | WF | MST |
| Syngeneic | | | 15 | | 10 |
| | 1 | 9 | | 9 | |
| | 2 | 19 | | 16 | |
| | 3 | 15 | | 15 | |
| | 4 | 18 | | 10 | |
| | 5 | 9 | | 9 | |
| Mixed xenogeneic (B10 + WF → B10) | | | 14 | | >53 |
| | 1 | 11 | | >53 | |
| | 2 | 14 | | >53 | |
| | 3 | 16 | | 16 | |
| | 4 | 9 | | 9 | |
| | 5 | 17 | | >53 | |
| Mixed xenogeneic (B10 + F344 → B10) | | | >53 | | 15 |
| | 1 | 19 | | >53 | |
| | 2 | >53 | | 13 | |
| | 3 | >53 | | 15 | |
| | 4 | >53 | | 15 | |
| | 5 | >53 | | 17 | |
| | 6 | 45 | | 14 | |
| | 7 | >53 | | 13 | |
| | 8 | >53 | | 13 | |
| | 9 | >53 | | 31 | |

The majority of mixed, xenogeneically reconstituted mice specifically rejected third-party rat. The two exceptions to this in which third-party rat was prolonged were probably due to a degree of immunoincompetence in these animals.

TABLE III

*Mixed Xenogeneic Chimeras: Cytotoxic Titer and Maximum Percent Kill of Sera Before and After Skin Grafting**

| Skin graft status | Animal | Prebleed‡ | Days after skin grafting | | | |
|---|---|---|---|---|---|---|
| | | | 13 | 22 | 36 | 45 |
| Rejected day 16 | Chimera A | 1:128 (65) | 1:32 (66) | 1:16 (66) | 1:16 (72) | 1:32 (71) |
| Intact >120 d | Chimera B | 1:32 (26) | 1:8 (5) | 1:2 (11) | 1:8 (15) | <1:2 |
| Intact >120 d | Chimera C | 1:8 (36) | 1:8 (6) | 1:2 (23) | <1:2 | <1:2 |
| Rejected day 105 | Chimera D | 1:512 (51) | <1:2 | <1:2 | <1:2 | 1:2 (10) |
| Rejected day 134 | Chimera E | 1:8 (18) | <1:2 | <1:2 (6) | 1:2 (17) | <1:2 |
| NA | Normal B10§ | <1:2 | <1:2 | <1:2 | <1:2 | <1:2 |

* Represented as titer (maximum percent kill) on F344 spleen target cells. All are corrected for percent complement kill.

‡ Prebleed refers to post-reconstitution but pre–skin grafting.

§ Mean ± SD of maximum percent kill for normal B10 serum = 4 ± 2.35.

TABLE IV

*Measurement of IgG1 and IgG2 Level Specific for F344 Lymphoid Cells by FMF**

| Sera | IgG1 | | | IgG2 | | |
|---|---|---|---|---|---|---|
| | Prebleed | Day 39 after skin graft | Δ | Prebleed | Day 39 after skin graft | Δ |
| Normal mouse | 25 | 24 | | 24 | 24 | |
| 1170 (mouse anti-rat) | 224 | 251 | | 1,440 | 1,352 | |
| Chimera | 60 | 55 | −5 | 115 | 28 | −8 |
| Chimera | 75 | 31 | −44 | 87 | 27 | −6 |
| Chimera | 24 | 30 | 6 | 26 | 27 | 1 |
| Chimera | 25 | 34 | 9 | 26 | 27 | 1 |
| Chimera | 100 | 28 | −72 | 140 | 28 | −16 |
| Chimera | 97 | 67 | −30 | 272 | 111 | −8 |

* Values are represented as total fluorescence units normalized to an arbitrary amplification value (GAIN 16). F344 rat splenocytes were stained with sequentially collected sera. Controls included normal mouse serum and a mouse anti-rat sera. All chimeric sera were from animals whose F344 rat grafts were intact for >39 d.

animal, chimera A, which rejected its F344 graft with a more rapid time course than most of the other animals. That animal demonstrated a relatively high titer and maximum percent kill, while the other animals exhibited a lower level of lysis similar to that for normal serum, while their skin grafts remained intact.

Because serologic enhancement could be one possible mechanism responsible for the prolonged acceptance of the F344 skin grafts, we examined the sera of experimental animals with grafts still intact for enhancing antibody. Because IgG1 and IgG2 have been demonstrated to be most commonly involved in enhancement (16), we directed our attention to analysis of levels of these two isotypes. Analysis of the sera from bleeds taken after reconstitution but before skin grafting and from day 50 after skin grafting revealed no increase in levels of gamma 1 or gamma 2 antibodies directed at F344 lymphoid cells by direct binding assay on the FACS using purified FITC-GAMIgG1 and IgG2 reagents. In fact, suppression of IgG1 and IgG2 levels was noted in some animals following skin grafting (Table IV).

In addition, an attempt was made to passively transfer such hyporeactivity to normal B10 animals. Serum from animals with rat skin grafts intact from 30 to 90 d was transferred (0.5 cc intravenously per normal B10) 4 h before placement of F344 rat and B10.D2 skin grafts. Control animals were unmanipulated. The rat and allogeneic skin grafts were rejected with identical time courses in the control and experimental animals (data not shown).

*Specific Tolerance to Donor-Type Lymphoid Elements as Evidenced by MLR.* As seen in a representative mixed lymphocyte culture (Table V), no response of chimeras to either host or donor-type stimulator cells was observed. Although the mixed xenogeneically reconstituted animals were somewhat suppressed in their overall responses, their responses to third party stimulators were present and specific. If one compares the ratios of response of the experimental animals to WF or B10.BR with that to F344, it can be seen that the ratios are much

TABLE V

*Host Hyporeactivity and Third-Party Reactivity of Mixed Xenogeneically Reconstituted Animals (B10 + F344 Rat → B10) in One-Way MLR**

| Animal | Anti-B10 | [$^3$H]Thymidine incorporation (cpm ± SEM) | | |
|---|---|---|---|---|
| | | Anti-F344 | Anti-WF | Anti-B10.BR |
| Normal B10 | 2,886 ± 920 | 31, 152 ± 2013 | 38,134 ± 4236 | 81,697 ± 8403 |
| Normal F344 | 135,734 ± 4108 | 4,710 ± 309 | 97,816 ± 4267 | 144,925 ± 7607 |
| Chimera 1 | 1,397 ± 94 | 1,575 ± 297 | 8,581 ± 260 | 17,105 ± 977 |
| Chimera 2 | 947 ± 128 | 1,645 ± 293 | 7,619 ± 1385 | 9,519 ± 537 |

* Mean ± SEM of triplicate cultures in 1:1 responder-to-stimulator ratio.

TABLE VI

*Search for Putative Suppressors: MLR*

| Responders ($4 \times 10^5$) | Actual values | | |
|---|---|---|---|
| | Additions | Anti-F344 | Anti-B10.BR |
| Normal B10 | 0 | 7,238 ± 495 | 2,438 ± 1216 |
| | $2 \times 10^5$ B10 | 12,470 ± 1066 | 5,720 ± 2075 |
| | $2 \times 10^5$ chimera 1 | 10,678 ± 577 | 9,586 ± 2188 |
| | $2 \times 10^5$ chimera 2 | 11,443 ± 832 | 10,366 ± 1602 |
| Normal B10 | 0 | 6,429 ± 795 | 4,168 ± 1897 |
| | $4 \times 10^5$ B10 | 11,861 ± 267 | 12,077 ± 6166 |
| | $4 \times 10^5$ chimera 1 | 11,699 ± 507 | 26,590 ± 735 |
| | $4 \times 10^5$ chimera 2 | 11,869 ± 482 | 25,353 ± 5693 |
| Chimera 1 | 0 | 1,278 ± 422 | 15,693 ± 1512 |
| Chimera 2 | 0 | 2,204 ± 165 | 21,004 ± 783 |
| | | Stimulation index* | |
| | Additions | Anti-F344 | Anti-B10.BR |
| Normal B10 | $2 \times 10^5$ chimera 1 | 1.5 | 2.9 |
| | $2 \times 10^5$ chimera 2 | 1.6 | 4.2 |
| | $2 \times 10^5$ normal B10 | 1.71 | 2.3 |
| | $4 \times 10^5$ chimera 1 | 1.8 | 6.4 |
| | $4 \times 10^5$ chimera 2 | 1.8 | 6.1 |
| | $4 \times 10^5$ normal B10 | 1.84 | 2.9 |

* Compared with normal B10 plus 2 or $4 \times 10^5$ normal B10 anti-rat responders.

greater than that of normal B10 to the same stimulators, which indicates specific tolerance to donor-type antigens.

The response of normal control B10 lymphocytes against F344 rat splenocytes in co-culture with chimeric cells has revealed no evidence for suppression (Table VI). Furthermore, titration of increasing numbers of co-cultured chimeric cells up to $1.6 \times 10^6$ cells with $4 \times 10^5$ normal B10 responding cells again failed to reveal evidence for suppression of the B10 anti-F344 response (data not shown). In addition, no suppression was seen in the reverse direction when F344 rat

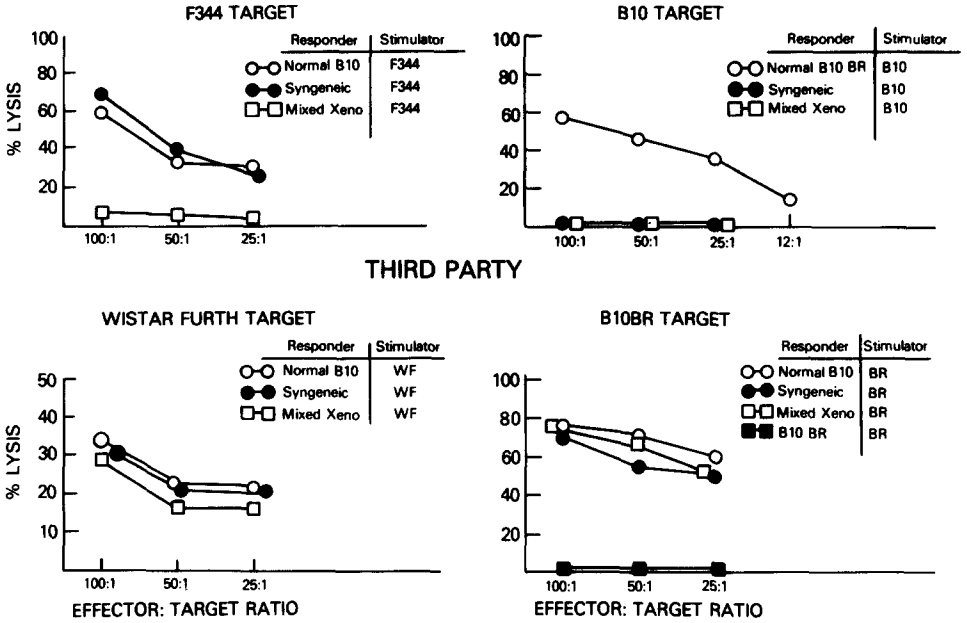


FIGURE 5. Specific CTL lysis of $^{51}$Cr-labeled target cells in one-way CML to host, donor, and third party targets. Spontaneous release was <25% unless otherwise indicated.

lymphocyte responding cells were co-cultured with unirradiated chimeric splenocytes and C57BL/10Sn stimulators (data not shown).

*Specific Tolerance to Donor-Type Lymphoid Elements as Evidenced by CML.* Cells from mixed xenogeneically reconstituted animals were examined in an in vitro CML sensitization. In one-way CML, responding cells were cultured with F344, B10, or third party murine or rat stimulators. Such attempts to sensitize (B10 + F344 → B10) spleen cells did not lead to cytotoxicity for either host (B10) or donor (F344)-type Con A– or PHA-stimulated blasts, but did lead to effective lysis of third-party mouse (B10.BR) and rat (WF) target cells (Fig. 5). This specific hyporeactivity persisted even after the onset of chronic rejection and eventual rejection of F344 skin grafts. Syngeneically reconstituted animals tested simultaneously demonstrated an anti-F344 and anti–third-party response at levels similar to normal B10 control responding cells. To detect suppression, normal B10 responder cells were co-cultured with unirradiated spleen cells from mixed xenogeneically resconstituted animals and F344 stimulators or third-party stimulators. In these experiments, co-culture of chimeric cells with normal B10 responder cells did not diminish the anti-F344 response or the anti–third-party response, and thus provided no evidence for specific or nonspecific suppression (Fig. 6).

*Immunocompetence as Assessed by PFC Responses.* In an attempt to further assess the in vivo immunocompetence of the mixed xenogeneically reconstituted animals, animals were subjected to intravenous immunization with SRBC suspensions and their ability to form PFC responses was quantitated. The responses of the mixed xenogeneically reconstituted mice were found to be immunocompetent and similar to syngeneically (B10 → B10) reconstituted mice in B cell and

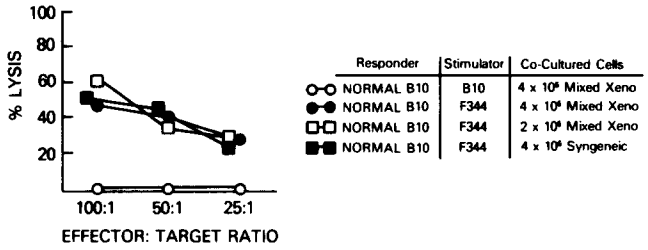


FIGURE 6. In an attempt to detect suppression, normal B10 responding cells were co-cultured with spleen cells from mixed xenogeneically reconstituted mice and F344 or third-party stimulators in CML. One representative co-culture experiment is presented. Target cells are $^{51}$Cr–PHA F344 lymph node blasts.

TABLE VII
*In Vivo SRBC PFC Response*

| Reconstitution | Number of animals | Plaques per $10^6$ spleen cells* | $P$‡ |
|---|---|---|---|
| Syngeneic (B10 → B10) | 9 | 62 (1.4) | — |
| Mixed xenogeneic (B10 + F344 → B10) | 5 | 38 (2.1) | <0.89 |
| Fully allogeneic§ (B10.D2 → B10) | 8 | 13.7 (2) | <0.08 |

* Data are presented as geometric means (standard error) of triplicate cultures read blind.
§ Unable to evaluate fully xenogeneically reconstituted mice due to poor survivals. Therefore, fully allogeneic chimeras were chosen as the most appropriate controls available.
‡ Significance of difference between the two experimental groups as compared with syngeneically reconstituted animals by two-tailed Student's $t$ test.

helper T cell interactions as assessed by their PFC responses. Fully xenogeneically reconstituted animals did not survive long enough for evaluation, and fully allogeneic chimeras were therefore chosen as the most appropriate available controls. As seen in Table VII, the majority of fully allogeneic chimeras (B10.D2 → B10) responded less well than did mixed xenogeneically reconstituted animals.

## Discussion

Animal models for fully xenogeneic chimeras have been less extensively investigated than those for allogeneic chimeras. This is in part due to the poor survival of such animals, presumably secondary to graft vs. host disease (17) and probable immunoincompetence due to a failure of appropriate immune cell interactions in the reconstituted host similar to that described in fully allogeneic chimeras (18, 19). In our experience, the absence of wasting disease and excellent survivals in the mixed xenogeneically reconstituted animals was in striking contrast to those animals reconstituted with identically treated T cell–depleted F344 bone marrow cells alone. It is difficult to assess whether the death of the fully xenogeneically reconstituted animals was due to graft vs. host disease or immunoincompetence. One can only conclude that their survival is poor but

prolonged over that of radiation controls if the animals are reconstituted with $>2.0 \times 10^7$ T cell–depleted F344 rat bone marrow cells.

It is apparent from our data that the persistence of a high level of donor-type lymphoid cells is not a requirement for prolonged in vivo and in vitro hyporeactivity to donor-type elements. In this aspect, the mixed xenogeneic model resembles neonatal tolerance as first described by Billingham et al. (1), in which only very small numbers of donor-type cells have been found (20). In fact, the percentage of rat cells detectable in peripheral lymphoid tissue was not a reliable prognosticator for the duration of skin xenograft prolongation or for the severity and temporal relationship of the chronic rejection reaction that eventually appeared in the skin grafts. The hyporeactivity to F344 rat antigens was manifested in varying degrees, depending upon the assay used. Although all mixed xenogeneically reconstituted animals manifested specific prolongation of skin xenograft survival, most grafts eventually underwent a chronic rejection and were slowly rejected. In contrast, in vitro specific hyporeactivity to F344 lymphocytes as assessed by MLR and CML was permanent and persisted even after the loss of F344 skin grafts following the prolonged chronic rejection.

Enhancement by serum factors has been proposed as one mechanism contributing to tolerance (16, 21). Voisin (21) reported no significant change in levels of IgG1 and IgA from pre–skin grafting to post–skin grafting in a neonatal murine chimera system, although both of these levels were elevated in comparison with normal control animals. In our system, the levels of IgG1 and IgG2 anti-rat antibody detectable in mixed xenogeneically reconstituted animals decreased or remained unchanged in animals retaining their rat tail skin grafts. However, before skin grafting, all animals had a degree of cytotoxic antibody directed against F344 splenic target cells after bone marrow grafting. In all animals that retained their xenografts for a prolonged period, the cytotoxic titer and maximum percentage lysis of donor-type target cells decreased or remained unchanged. In contrast, the titer and maximum lysis by serum from the one animal that rejected its skin graft rapidly remained elevated. These data could be suggestive of a possible humoral factor contributing to the specific hyporeactivity observed in vivo and in vitro in mixed xenogeneically reconstituted animals, although attempts to demonstrate enhancement with serum from hyporeactive animals were unsuccessful. Serum antibody could also represent a lack of tolerance at the humoral level to donor-type xenogeneic lymphoid elements. In either case, specific hyporeactivity to skin xenografts and to donor-type lymphoid antigens in vitro appear in this model to occur in the presence of specific antibody production to F344 lymphoid cells.

In search of putative active suppression, we performed mixing experiments in which chimeric lymphocytes were co-cultured with normal B10 or normal F344 responding cells and B10, F344, or third-party stimulators in mixed lymphocyte culture. We chose mixed reconstituted animals with skin grafts still intact with the presumption that the strong antigenic stimulus of an intact skin graft might expand a possible suppressor cell population. However, neither specific nor nonspecific suppression of the proliferative reaction in MLR and cytotoxic lymphocyte reaction in CML could be demonstrated in numerous experiments.

In addition, no suppression of the F344 rat anti-B10 MLR response was noted in co-culture experiments.

Although small percentages of donor-type cells have been identified in various lymphoid compartments of some of the mixed xenogeneically reconstituted animals, many animals have had no donor-type cells detectable. However, all animals have demonstrated prolongation of donor-type skin graft survival beyond that of syngeneically reconstituted controls. Because skin is strongly antigeneic and the survival of skin grafts is frequently more difficult to prolong than most other solid organs (22), it seems likely that animals manifesting this level of hyporeactivity would be highly tolerant to xenografts of kidney, heart, or other vascularized organs. Studies are currently in progress involving heterotopic caridac xenograft transplantation in such animals to assess this possibility.

This new approach may provide a model for investigation of mechanisms of self-recognition and antigen processing in addition to that of transplantation tolerance. While clinical applications of mixed xenogeneic bone marrow reconstitution would be premature, the approach may offer advantages for bone marrow and cadaver donor organ transplantation. Single high-dose radiation and immediate mixed reconstitution coincident with solid organ transplantation could, for example, afford immediate use of donor organs with potential for prolonged acceptance of the donor allograft or xenograft in the absence of prolonged nonspecific chemical immunosuppressive agents. Attempts to extend these studies to a large animal model are in progress.

## Summary

Mixed xenogeneically reconstituted mice (F344 rat + C57BL/10Sn → C57BL/10Sn), which specifically retain F344 tail skin xenografts, were studied for the specificity of such hyporeactivity and for in vitro reactivity and immunocompetence. Survival of mixed reconstituted animals was excellent, without evidence for graft vs. host disease. Donor-type tail skin grafts were specifically prolonged (mean survival time = 80 d) in comparison with normal controls and syngeneically reconstituted animals. In vitro, such animals manifested specific hyporeactivity by mixed lymphocyte reaction and cell-mediated lympholysis to F344 rat and B10 cells, with normal response to third-party rat (Wistar-Furth) and mouse (B10.BR). Examination of lymphoid tissues with a fluorescence-activated cell sorter revealed low levels, if any, of donor-type cells detectable. This system offers a model for investigation of xenogeneic transplantation tolerance.

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