TRANSPLANTATION TOLERANCE IN ADULT RATS USING TOTAL LYMPHOID IRRADIATION: PERMANENT SURVIVAL OF SKIN, HEART, AND MARROW ALLOGRAFTS*

By S. SLAVIN,‡ B. REITZ, C. P. BIEBER, H. S. KAPLAN, AND S. STROBER§

(From the Division of Immunology, Department of Medicine, the Department of Cardiovascular Surgery, and the Division of Radiotherapy, Department of Radiology, Stanford University School of Medicine, Stanford, California 94305)

Establishment of permanent tolerance to transplantation antigens remains the major obstacle in clinical organ transplantation. The rejection phenomenon persists despite extensive use of a large variety of immunosuppressive protocols that include radiation, cytotoxic agents, corticosteroids, and anti-lymphocyte antisera. Some interesting experimental approaches to organ transplantation are currently under investigation (1–4). However, none of these is clinically applicable at present.

Recently, a new approach was developed for transplanting bone marrow (BM)[1] allografts across major histocompatability barriers with induction of permanent and specific tolerance to donor BM-type alloantigens in mice (5–7). Permanent (>300 days) chimerism was established in adult BALB/c (H-$2^{d/d}$) mice after treatment with total lymphoid irradiation (TLI), and infusion of 10^7 C57BL/Ka (H-$2^{b/b}$) BM cells (7). TLI consisted of 17 consecutive fractions of 200 rads (total cumulative dose of 3,400 rads) directed to the major lymphoid organs, including the thymus, spleen, and cervical, axillary, mediastinal, inguinal, and mesenteric lymph nodes. A lead shield covered the skull, lungs, ribs, thoracic spine, hind legs, and tail (5, 7). A similar radiotherapy technique is routinely used in humans for the treatment of Hodgkin's disease (8). The specificity of the acquired tolerance to the H-2^b haplotype in the H-2^d recipients was demonstrated by the normal rejection pattern of a third-party skin allograft of H-2^k haplotype (C3H/He) with permanent survival of an adjacent H-2^b (C57BL/Ka) graft (7). A series of in vitro experiments revealed that lymphocytes from BM chimeras lost their ability to respond against either donor- or recipient-type lymphocytes in a one-way mixed lymphocyte reaction (MLR) (7). Interestingly, BM-recipient BALB/c mice did not develop clinical signs of graft-versus-host disease (GVHD) (7). The present work confirms and extends

* Supported by National Institutes of Health grants AI 11313, CA 17004, CA 05838, and HL 08696-14.

‡ Recipient of Fogarty International Fellowship 5FO5 TW 02227, on leave from the Department of Medicine A, Hebrew University, Hadassah Medical Center, Jerusalem, Israel.

§ Investigator, Howard Hughes Medical Institute.

[1] *Abbreviations used in this paper:* BM, bone marrow; BN, Brown Norway rats; GVHD, graft-versus-host disease; MLR, mixed lymphocyte reaction; TLI, total lymphoid irradiation.

previous observations in mice. Adult rats treated with TLI and given infusions of allogeneic BM permanently accepted heterotopically transplanted skin and perfused heart allografts. BM chimeras were specifically tolerant to donor tissues, and showed no clinical signs of GVHD.

Materials and Methods

Animals and Irradiation. Adult male Lewis ($AgB^{1/1}$), Brown Norway (BN) ($AgB^{3/3}$) and ACI ($AgB^{4/4}$) rats purchased from Microbiological Associates, Walkersville, Md., were used in all experiments. Rats weighed 250–350 g. Animals were irradiated with a Philips x-ray unit (250 kV, 15 mA; Philips Electronic Instruments, Inc., Mahwah, N.J.) at a rate of 40 rads/min with 0.25-mm Cu and 1.0-mm Al filters. The source axis distance was 60 cm. Lewis allograft recipients were treated with 200 rads/day, five times a week to achieve a total dose of 3,400 rads before transplantation. Animals were anesthetized with pentobarbital and positioned in an apparatus with lead shields (Fig. 1) for each radiation treatment. Two radiation protocols were used; one (Fig. 1 A) shielded the skull, lungs, limbs, pelvis, and tail; the other (Fig. 1 B) shielded the skull, lungs, and tail, but left the entire pelvis and the forelimbs exposed to x-rays. In both cases, all the major lymphoid organs, including the thymus and spleen, were in the radiation fields. Dosimetry was verified using a calibrating ionization chamber, and lithium fluoride thermoluminescence dosimeters.

Skin, BM, and Heart Transplantation. Full-thickness skin grafts were prepared using a modification of the technique of Billingham and Medawar (9), and they were transplanted to the ventral thoracic wall. Grafts were examined daily, and rejection was considered complete when they had sloughed off, or an eschar had developed. BM cells obtained from the long bones were made into a single cell suspension, and injected into the lateral tail vein of irradiated recipients as described previously (7). Heart allografts were anastomosed to the host's abdominal aorta and inferior vena cava according to the method of Ono and Lindsey (10). Cardiac allograft survival was monitored by palpation and electrocardiography. Grafts were considered rejected when both palpable contractions and electrocardiographic activity were no longer detected. Skin, BM, and heart allografts were transplanted to Lewis recipients within 1 day after the completion of TLI.

Assay for Chimerism. Survival of BM allografts was measured by the persistence of donor-type lymphocytes in the peripheral blood. Donor lymphocytes were detected by an in vitro complement-dependent microcytotoxicity assay (7) using anti-AgB antisera. Peripheral blood lymphocytes purified on a Ficoll-Hypaque gradient (11) were incubated for 15 min at room temperature with diluted antiserum in medium 199 (Microbiological Associates) with 5% heat-inactivated fetal calf serum. Rabbit complement, thoroughly absorbed with rat spleen cells (1:1 for 30 min on ice) was added (final dilution 1:5), and the reaction mixture was incubated for another 45 min at room temperature. Thereafter, cells were harvested by centrifugation, and resuspended in culture medium. The percentage of cytotoxicity was determined after the addition of trypan blue. The net cytotoxicity index was calculated by comparing the number of viable cells present after treatment with antiserum to the number present after treatment with normal rat serum. The percentage of donor-type lymphocytes in Lewis recipients was always cross-checked by determining the percentage of host-type lymphocytes. In all cases the sum approximated 100 $\pm$ 10%.

Preparation of Antisera. Specific Lewis anti-ACI, Lewis anti-BN, BN anti-Lewis, and ACI anti-Lewis antisera were raised by immunizing normal recipients with a skin allograft, and injecting 50×10^6 allogeneic spleen cells intraperitoneally 2 wk after graft rejection. Recipients were bled 1 wk later. The antisera were found to kill 100% of the allogeneic lymphocytes, and <5% of syngeneic lymphocytes in the microcytotoxicity test at a dilution of 1:40.

MLR. To document specific transplantation tolerance, peripheral blood lymphocytes from Lewis recipients were stimulated with allogeneic lymphocytes from the BM donor and third-party strains in the one-way MLR. Cultures contained 0.1×10^6 responding cells and 0.2×10^6 irradiated (3,000 rads, in vitro) stimulator cells in 0.2-ml round-bottomed microculture plates using 10% heat inactivated human AB serum. Cells were incubated at 37°C in a 5% CO_2-air mixture for 72 h, and then pulsed for 16–18 h with 1 mCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.). Details of the assay were described previously (7).

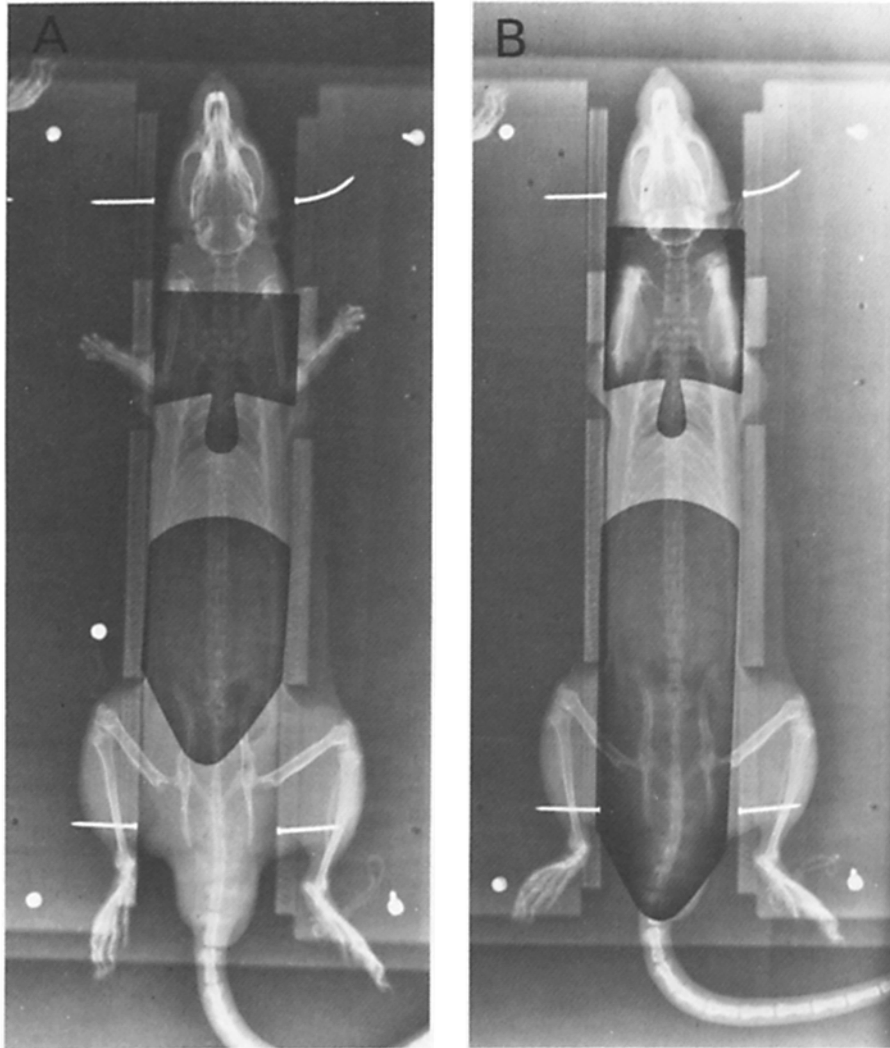

FIG. 1. Double exposure roentgenogram of anesthetized rats in apparatus used for irradiation of major lymphoid tissues. Opaque areas overlying animals are lead shields. All tissues protruding from rectangular enclosure were also shielded (shields not shown). Radiation ports were confined to lucent areas within rectangular enclosures. (A), Most of pelvis and forelimbs are covered by shields; (B), whole of pelvis and forelimbs are exposed to irradiation.

Results and Discussion

Lewis rats were given TLI with pelvic and forelimb shields. Skin allografts were transplanted 1 day after irradiation was complete. Lewis recipients showed markedly delayed rejection of BN and ACI skin allografts (mean survival was 39 and 41 days respectively) as compared to unirradiated controls (of mean survival 10 days; Table I). Rejection of BN and ACI heart allografts was even further delayed (survival range, 34–>300 days) as compared to controls (survival range, 9–10 days; Table II).

None of the Lewis rats given TLI with pelvic and forelimb shields developed

TABLE I
Survival of ACI and BN Skin Allografts on Lewis Rats

Treatment of Lewis recipients	Skin allograft donor	Number of rats	Skin allograft mean survival
			days (range)
TLI*	BN	6	39 (33-46)
TLI*	ACI	6	41 (35-47)
TLI* + 10^7 BM cells (BN)	BN	5	43 (36-65)
TLI* + 1-2 × 10^8 BM cells (BN)	BN	2	44 (39-49)
TLI* + 1-3 × 10^8 BM cells (ACI)	ACI	5	32 (21-44)
TLI (without pelvic and forelimb shields) + 10^8 BM cells (ACI)	ACI	5	>150 (40->150)
None	BN	4	10 (9-11)
None	ACI	4	10 (10)

* TLI with pelvic and forelimb shields.

TABLE II
Survival of ACI and BN Heart Allografts in Lewis Rats

Treatment of Lewis recipients	Heart allograft donor	Number of rats	Heart allograft survival
			days
TLI*	BN	3	35, 66, 70
TLI*	ACI	3	77, 80, >300
TLI* + 10^8 BM cells (ACI)	ACI	5	145, >300, >300, >300, >300
None	ACI	3	9, 9, 9
None	BN	5	9, 9, 9, 9, 10

* TLI with pelvic and forelimb shields.

TABLE III
Survival of ACI and BN Bone Marrow Allografts in Lewis Rats

Treatment of Lewis recipients	Fraction of definite chimeras >100 days after transplantation*	Mean of donor-type lymphocytes in peripheral blood
		%
TLI§ + 10^7 BM cells (BN)	0/7	0
TLI§ + 2 × 10^8 BM cells (BN)	0/2	0
TLI§ + 3 × 10^8 BM cells (BN)	0/2	3
TLI§ + 10^8 BM cells (ACI)	0/5	7
TLI§ + 2 × 10^8 BM cells (ACI)	0/1	6
TLI§ + 3 × 10^8 BM cells (ACI)	0/2	0
TLI (without pelvic and forelimb shields) + 10^8 BM cells (ACI)	2/4	48‡
TLI (without pelvic and forelimb shields) + 3 × 10^8 BM cells (ACI)	4/4	57‡

* >10% donor-type peripheral blood lymphocytes was considered evidence of definite chimerism.
‡ Mean of individual values of definite chimeras in each group.
§ TLI with pelvic and forelimb shields.

definite stable chimerism (e.g. >10% donor type cells) of the peripheral blood lymphocytes after the intravenous injection of up to 3 × 10^8 ACI or BN marrow cells (Table III). However, Lewis recipients given TLI without pelvic and forelimb shields accepted ACI bone marrow allografts, and exhibited definite

TABLE IV
MLR of Peripheral Blood Lymphocytes of Lewis Rats

Treatment of Lewis donors of responding cells	Treatment of donors of stimulating cells	Strain of donors of stimulating cells	[^{3}H]Thymidine uptake*
			cpm ± SD
None	None	Lewis	382 ± 165
None	None	ACI	9,247 ± 329
None	None	BN	12,267 ± 1,462
None	TLI‡ + 10^8 BM cells (ACI) (bearing long-term heart graft)	Lewis	727 ± 367
None	TLI§ + 10^8 BM cells (ACI) (bearing long-term skin graft)	Lewis	9,867 ± 2,053
TLI‡ + 10^8 BM cells (ACI) (bearing long-term heart graft)	None	Lewis	471 ± 152
TLI‡ + 10^8 BM cells (ACI) (bearing long-term heart graft)	None	ACI	804 ± 159
TLI‡ + 10^8 BM cells (ACI) (bearing long-term heart graft)	None	BN	2,202 ± 159
TLI§ + 10^8 BM cells (ACI) (bearing long-term skin graft)	None	Lewis	269 ± 140
TLI§ + 10^8 BM cells (ACI) (bearing long-term skin allograft)	None	ACI	197 ± 6
TLI§ + 10^8 BM cells (ACI) (bearing long-term skin graft)	None	BN	1,203 ± 209

* Mean ± SD of triplicate values. One of two experiments is shown.
‡ TLI with pelvic and forelimb shields.
§ TLI without pelvic and forelimb shields.

chimerism of the peripheral blood lymphocytes in assays performed 3–10 mo after marrow transplantation (Tables III, IV). Successful engraftment was also a function of the number of infused BM cells (Table III). Although the marrow donors and recipients differed at the major histocompatability genetic region (AgB), no clinical evidence of GVHD (e.g. diarrhea, hunched back, loss of fur) was observed in the chimeras. A similar lack of GVHD was found previously in chimeric BALB/c mice given TLI and C57BL/Ka BM cells (7.

All of the Lewis recipients given TLI with pelvic and forelimb shields, and ACI or BN marrow cells, rejected allogeneic skin grafts within 65 days (Table I). However, 4 out of 5 Lewis recipients given TLI without pelvic and forelimb shields, and 10^8 ACI marrow cells accepted ACI skin grafts for more than 150 days (Table I). The latter four animals were found to be stable chimeras. Definite chimerism was not observed in the one rat that rejected the skin graft after 40 days. Two of the Lewis rats bearing ACI skin grafts for more than 150 days, were given BN skin allografts. These third-party skin grafts were

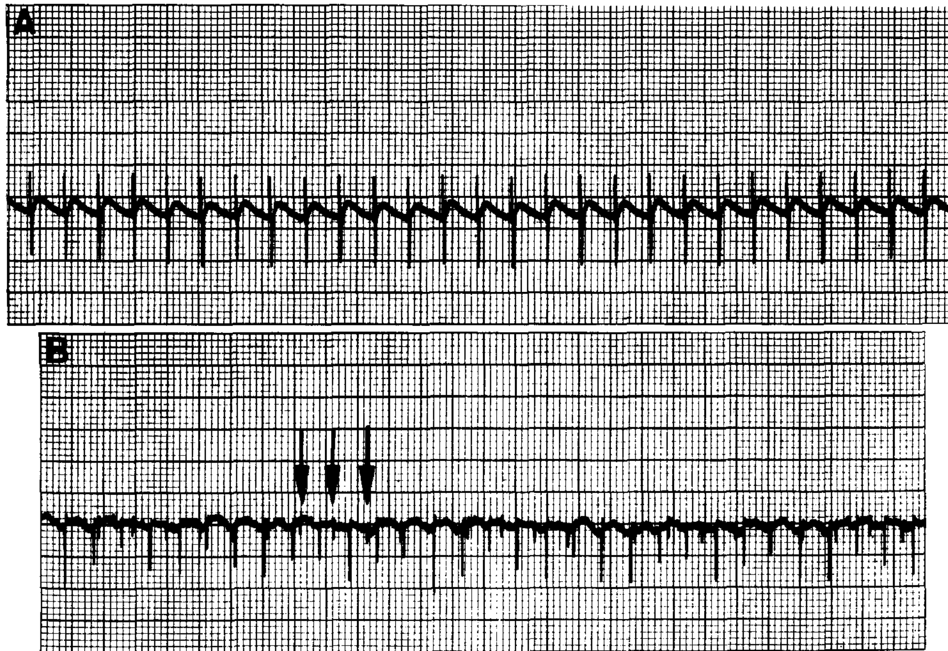

FIG. 2. (A) Electrocardiogram with V-lead recording from area of abdomen overlying heterotopic heart allograft transplanted 360 days earlier. (B) Electrical activity of both host and donor (arrow) hearts are shown.

rejected within 25 days. This shows that chimeric Lewis rats were specifically tolerant to the ACI tissue transplantation antigens.

We were unable to demonstrate definite stable chimerism in Lewis rats given TLI, with pelvic and forelimb shields, and ACI marrow cells. However, 4 out of 5 nonchimeric rats accepted ACI heart allografts permanently (>300 days; Table II). The electrical activity of one of these long-term heart allografts is shown in Fig. 2. ACI skin allografts transplanted to Lewis rats bearing ACI heart allografts for 300 days were rejected within 20 days. Nevertheless, the heart allografts persisted for at least 60 days after the rejection of the skin grafts. Preferential acceptance of heart as compared to skin allografts in rats has been described previously in several model systems (1, 12). Prolonged heart allograft survival in the present experiments could be explained by several mechanisms including the development of specific suppressor cells, enhancing antibodies, and/or low levels of chimerism undetected by our assays.

Peripheral blood lymphocytes from chimeric Lewis rats bearing ACI skin grafts showed no reactivity to ACI or Lewis stimulator cells in the MLR assay (Table IV). Although the response to BN cells was considerably greater than that to the Lewis or ACI cells, it was markedly reduced in comparison to the response of normal Lewis rats (Table IV). This shows that nonspecific, as well as specific depression of the in vitro immune response persists for at least several months in the chimeras. A similar pattern of MLR reactivity was also observed in Lewis rats bearing long-term ACI heart allografts (Table IV). The lack of chimerism in the latter recipients was further documented by the

inability of their peripheral blood lymphocytes to stimulate responder cells from normal Lewis rats. On the other hand, peripheral blood lymphocytes from Lewis recipients bearing long-term ACI skin allografts vigorously stimulated normal Lewis responder cells.

The present work extends earlier observations concerning the induction of tissue transplantation tolerance in a single strain combination (C57BL/Ka → BALB/c) of mice using TLI. The results reported herein show that successful transplantation of allogeneic BM without GVHD can be achieved in rats as well as in mice, and that TLI can be used to obtain permanent survival of perfused organ allografts. Development of stable BM chimerism in rats required the use of radiation ports similar to those used in mice (e.g., inclusion of the pelvis and forelimbs), and transfer of adequate numbers of BM cells. This suggests that extensive marrow as well as lymphoid irradiation is required for successful allogeneic marrow engraftment. Radiotherapy techniques similar to those described above are routinely used to treat lymphoid malignancies in humans, and have been shown to be relatively safe (8). These techniques have considerable potential application to clinical BM and perfused organ transplantation.

Summary

Lewis rats given total lymphoid irradiation (TLI) accepted bone marrow allografts from AgB-incompatible donors. The chimeras showed no clinical signs of graft-versus-host disease. Skin allografts from the marrow donor strain survived for more than 150 days on the chimeras. However, third-party skin grafts were rejected promptly. Although heart allografts survived more than 300 days in Lewis recipients given TLI and bone marrow allografts, detectable levels of chimerism were not required for permanent survival.

We thank G. Garrelts and C. M. Johnson for their valuable technical assistance.

Received for publication 7 November 1977.

References

1. Dittmer, J., and M. Bennett. 1975. Long-term survival of cardiac allografts in lethally irradiated rats repopulated with host-type hemopoietic cells. *Transplantation (Baltimore).* **19**:295.
2. Marguet, G., and G. Heystek. 1975. The induction and abolition of specific immunosuppression of heart allografts in rats by use of donor blood and cyclophosphamide. *J. Immunol.* **115**:405.
3. Binz, H., and H. Wigzell. 1976. Specific transplantation tolerance induced by autoimmunization against the individual's own, naturally occurring idiotypic, antigen-binding receptors. *J. Exp. Med.* **144**:1438.
4. Muller-Rucholtz, W., H. U. Wottge, and H. K. Muller-Hermelink. 1976. Bone marrow transplantation in rats across strong histocompatibility barriers by selective elimination of lymphoid cells in donor marrow. *Transplant. Proc.* **8**:537.
5. Slavin, S., S. Strober, Z. Fuks, and H. S. Kaplan. 1976. Long-term survival of skin allografts in mice treated with fractionated total lymphoid irradiation. *Science (Wash. D. C.).* **193**:1252.

6. Slavin, S., S. Strober, Z. Fuks, and H. S. Kaplan. 1977. Use of total lymphoid irradiation in tissue transplantation in mice. *Transplant. Proc.* 9:1001.
7. Slavin, S., S. Strober, Z. Fuks, and H. S. Kaplan. 1977. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *J. Exp. Med.* 146:34.
8. Kaplan, H. S. 1972. Hodgkin's Disease. Harvard University Press, Cambridge, Mass. 216–279.
9. Billingham, R. E., and P. B. Medawar. 1951. The technique of free skin grafting in mammals. *J. Exp. Biol.* 28:385.
10. Ono, K., and E. Lindsey. 1969. Improved technique of heart transplantation in rats. *J. Thorac. Cardiovasc. Surg.* 57:225.
11. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. Suppl.* 21:77.
12. Warren, R. P., J. S. Lofgreen, and D. Steinmuller. 1973. Differential survival of heart and skin allografts in inbred rats. *Transplant. Proc.* 5:717.