INDUCTION OF SPECIFIC TISSUE TRANSPLANTATION TOLERANCE USING FRACTIONATED TOTAL LYMPHOID IRRADIATION IN ADULT MICE: LONG-TERM SURVIVAL OF ALLOGENEIC BONE MARROW AND SKIN GRAFTS*

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In 1953, Medawar and his colleagues (1) induced tissue transplantation tolerance in mice by injecting allogeneic bone marrow cells intravenously into newborn recipients. Permanent chimerism was established without runt disease. Recipients accepted donor skin grafts but rejected third-party grafts (2). These experimental results were predicted by Burnet and Fenner (3).

Although chimerism can be established by injecting allogeneic bone marrow into lethally irradiated adult mice, a lethal graft vs. host disease ensues in most strains of mice (4). Even in those strains in which minimal graft vs. host disease is observed, late deaths (>100 days after transplantation) occur in a high proportion (up to 40%) of recipients (5). Recently, stable chimerism and specific tolerance have been induced in adult mice and rats by lethal whole body irradiation of recipients followed by the intravenous infusion of allogeneic fetal liver cells (6) or adult bone marrow cells treated in vitro to remove thymus-derived (T) cells and/or T-cell precursors (7, 8). Little or no clinical evidence of graft vs. host disease was noted in the latter studies. In addition, adult rats have been rendered tolerant to allogeneic cells after treatment with anti-idiotype antibody directed against receptor sites for AgB alloantigens (9).

Successful clinical application of techniques developed for the induction of transplantation tolerance in laboratory rodents requires that: (*a*) the recipient treatment can be relatively safe in humans, (*b*) the technique can be used in outbred species, and (*c*) long-term survival of allogeneic organ grafts can be achieved. Thus, animal models of tissue tolerance induction which depend upon lethal irradiation may not be practical for clinical organ transplantation due to severe and sometimes fatal side effects of such treatment (10, 11). The requirement for the use of inbred animals as donors of large numbers of fetal cells and organ grafts (6, 8), or as transplant recipients (7) is also impractical. Finally, those tolerization regimens which prolong organ graft survival in rodents for only 1–2 mo (9), do not meet the goals of long-term graft survival in humans.

The present report describes a new technique for the induction of specific transplantation tolerance in mice using fractionated total lymphoid irradiation

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(TLI).[1] This radiotherapy regimen is used in the treatment of human malignant lymphomas, and is associated with few severe side effects (12). Mice treated with TLI accepted *H-2* incompatible bone marrow grafts for at least 250 days without clinical evidence of graft vs. host disease in a completely allogeneic strain combination. Specific long-term tissue tolerance was documented in the stable chimeras by transplantation of appropriate skin allografts, and by examination of the in vitro response to allogeneic lymphocytes [mixed leukocyte reaction (MLR)]. The possible application of these findings to clinical organ transplantation is discussed.

Materials and Methods

Animals. Inbred male and female BALB/c ($H\text{-}2^{d/d}$) mice, 6- to 8-mo old, were used as irradiated recipients of allografts. Female C57BL/Ka ($H\text{-}2^{b/b}$), C3H/He ($H\text{-}2^{k/k}$), and (BALB/c × C57BL/Ka)F_1 ($H\text{-}2^{d/b}$) mice were the donors of bone marrow cells and allogeneic skin grafts. All mice were bred in pathogen-free conditions in the Division of Radiobiology, Department of Radiology, Stanford University, Stanford, Calif., but were kept in conventional housing during all experiments.

Radiation Source. X rays were delivered by a Philips unit (250 kV, 15 mA; Philips Medical Systems, Inc., Mount Vernon, N. Y.) at a rate of 40 rads/min. The source to skin distance was 60 cm, and 0.25 mm Cu plus 1.0 mm Al filters were used. Dosimetry was verified using a calibrating ionizing chamber, and by lithium fluoride thermoluminescence dosimeters.

Radiation Procedure. BALB/c mice were anesthetized with pentobarbital for proper positioning in an apparatus that was designed to expose the major lymph nodes (submandibular cervical, axillary, inguinal, and mesenteric), the thymus, and the spleen. Most of the skull, ribs, lungs, hind legs, and tail were shielded with lead (Fig. 1). Unless otherwise noted, all mice received 200 rads/day five times a week to a total dose of 3,400 rads.

Skin Grafting. A full thickness skin graft was transplanted to the flank of several groups of irradiated and control mice (13). The grafts were followed by daily inspection and palpation. Rejection was taken as the time of complete sloughing, or when a dry scab was formed.

Splenectomy. In some experiments, splenectomy was performed 3 wk before the irradiation regimen to allow for complete recovery from the surgical procedure. The spleen was exposed through a midline abdominal incision and the pedicle tied off with 4-0 silk. The pedicle was severed distally, and the incision was closed with silk sutures.

Peripheral Blood. Mice were bled periodically from the retro-orbital veins. Blood was collected and pooled from groups of experimental and control mice using sterile glassware coated with preservative-free heparin.

Purification of Peripheral Blood Lymphocytes (PBL). Peripheral blood was collected into dry heparinized glass tubes, and diluted 1:5 (vol/vol) with phosphate-buffered saline (PBS). The suspension was layered on a Ficoll-Hypaque gradient (14). After centrifugation for 40 min at room temperature, cells were aspirated from the PBS-Ficoll interface and washed in PBS.

PBL Counts. Peripheral blood was diluted in acetic acid (2%) and nucleated cells were counted in a standard hemocytometer. Absolute PBL counts were calculated by multiplying the percentage of lymphocytes in blood smears treated with Wright's strain, and the nucleated (white) blood cell count. Mean normal values for PBL, and leukocytes (WBC) were obtained from pooled blood samples taken from a single group of 12 control mice on 14 different occasions during a 6 mo period.

Quantitation of T Lymphocytes. The absolute T-cell count in the peripheral blood was calculated from the percentage of T lymphocytes, and the absolute lymphocyte count. The percentage of T cells was determined using a dye-exclusion microcytotoxicity test with a noncongenic anti-Thy 1.2 antiserum (AKR/J anti-C3H/He thymocyte antiserum) (15). Ficoll-Hypaque-purified PBL were washed twice in PBS, and contaminating erythrocytes (RBC) were lysed with ammonium chlo-

[1] *Abbreviations used in this paper:* BM, bone marrow; Con A, concanavalin A; FCS, fetal calf serum; MLR, mixed leukocyte reaction; m.s.t., mean survival time; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; RBC, red blood cells; TLI, total lymphoid irradiation; WBC, white blood cells.

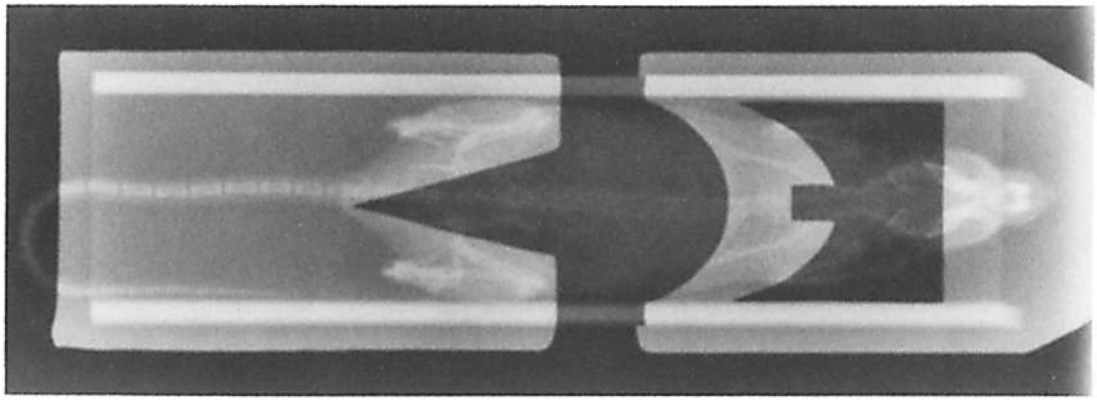

FIG. 1. A double exposure X-ray film of an anesthetized mouse in a lead apparatus designed for exposure of the major lymphoid organs to irradiation.

ride. The lymphocytes were resuspended in medium 199 containing 5% heat-inactivated fetal calf serum (FCS) to a concentration of 2×10^6 ml. 10 μl of the lymphocyte suspension was incubated with 10 μl of anti-Thy 1.2 antiserum (1:10) for 30 min at room temperature. 5 μl of guinea pig complement (C') (final concentration 1:5) was added for an additional 45 min at room temperature. The cells were spun at 250 *g* for 5 min and resuspended in 15 μl of medium 199. Trypan blue was added before each reading.

Quantitation of Immunoglobulin (Ig)-Bearing Lymphocytes (B Lymphocytes). Absolute B-cell counts in the peripheral blood were calculated from the percentage of B lymphocytes, and the absolute lymphocyte count. Ig-bearing cells were identified by a two-stage immunofluorescent-staining procedure using a polyvalent rabbit-anti-mouse Ig,[2] and a fluorescein-conjugated goat-anti-rabbit Ig antiserum (Meloy Laboratories Inc., Springfield, Va.) as described previously (16). PBL were stained in suspension, and smeared onto glass slides. The percentage of fluorescent cells was determined after counting a total of 100–200 small cells at 400 magnification using a Zeiss microscope (Carl Zeiss, Inc., New York) with a tungsten light source and fluorescein isothiocyanate excitation filter.

MLR. The proliferative response of pooled PBL from normal and irradiated BALB/c mice to allogeneic stimulator cells was measured at different times after TLI (17). 1×10^6 responding cells were cultured in 0.2 ml flat bottom microculture wells (Microtest II tissue culture plate, Falcon 3040; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) with 1×10^6 stimulator lymphocytes isolated from peripheral lymph nodes. Stimulator cells were inactivated by a single in vitro exposure to 3,400 rads from a radioactive cesium-137 source (Mark I model 25 irradiator; J. L. Shepherd and Associates, Glendale, Calif.). Irradiated syngeneic lymph node cells served as stimulator cells in negative controls for each experiment. The culture medium contained RPMI 1640 medium supplemented with human AB serum (10%), glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 U/ml), and streptomycin (100 μg/ml). Tissue culture media and reagents were purchased from Grand Island Biological Co., Grand Island, N. Y. Cells were incubated 37°C with 5% CO_2-air mixture in a humidified incubator for 72 h, and then pulsed for 16–18 h with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) per culture. Cells were subsequently collected on paper filters using a multiple sample harvester (Mash II; Microbiological Associates, Inc., Bethesda, Md.). Filters were dried, dispensed into vials containing 3 ml of scintillation fluid, and counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The MLR of lymph node cells from allogeneic bone marrow chimeras was assayed using 0.5×10^6 responding cells and 1×10^6 stimulating cells in 0.2 ml round bottom microculture wells (Microtiter plates, 1-220-24 A; Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). Otherwise, the MLR procedure was identical to that described above.

[2] Polyvalent rabbit-anti-mouse Ig was kindly supplied by Dr. L. A. Herzenberg, Department of Genetics, Stanford University School of Medicine, Stanford, Calif.

Blastogenic Response to Phytohemagglutinin (PHA). 2×10^5 purified PBL from irradiated mice were aliquoted into 0.2 ml microculture wells containing RPMI 1640 supplemented with FCS (10%), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). PHA (Purified-Phytohemagglutinin; Burroughs-Wellcome, London, England) was added at the optimal concentration of 0.6 μg/ml. Cultures were done in triplicate. PBL from age- and sex-matched normal mice were used as controls in each experiment. The cultures were pulsed with 1 μCi of [^{3}H]thymidine after 72 h, and uptake was measured as described in the previous section. Results were expressed as the percentage of the normal response (cpm experimental/cpm normal × 100) in order to correct for considerable variability in the absolute [^{3}H]thymidine uptake (cpm) in controls in different experiments during the long observation period. Background values for experimental and control groups were less than 1,000 cpm.

Blastogenic Response to Concanavalin A (Con A). 4×10^5 purified PBL from irradiated mice were added to microculture wells in 0.2 ml tissue culture medium as described in the previous section. Con A (Pharmacia Fine Chemicals, Ltd., Uppsala, Sweden) was added at an optimal concentration of 2.5 μg/ml. PBL from age- and sex-matched normal mice were assayed concomitantly, and all cultures were done in triplicate. [^{3}H]thymidine uptake was measured as before, and results were expressed as the percentage of the normal response (cpm experimental/cpm control × 100). Background values for experimental and control groups were less than 1,000 cpm.

Infusion of Bone Marrow (BM) Cells into BALB/c Mice Given TLI. BM cells were obtained by flushing minimal essential medium (MEM) through the shafts of the femori and tibiae of donor mice. Cells were washed once and resuspended in MEM. Aliquots of 0.25 ml medium containing 10^7 nucleated cells were injected intravenously to recipient mice.

Assays for Chimerism

CHIMERISM OF LYMPHOCYTES. Chimerism of PBL, spleen, lymph nodes, and BM was assayed using an in vitro microcytotoxicity test (18) to determine the percentage of lymphocytes carrying host or donor *H-2* surface markers. Lymphocytes from all of the above sources were purified on a Ficoll-Hypaque gradient, washed three times in PBS with 5% FCS, and resuspended at 1×10^6 cells/ml in PBS with 5% FCS. 2 μl of the cell suspensions, and 2 μl of specific anti-H-2 antiserum diluted in PBS were incubated in microtest plates (Falcon 3034; Falcon Plastics, Div. of BioQuest) for 15 min in a humidified atmosphere at 37°C. Cells were washed once with cold, serum-free PBS, and incubated for 30 min at 37°C in 2 μl of rabbit C′ diluted 1:6 with PBS. The reaction was terminated by the addition of 25 μl cold PBS containing 5% FCS, and Nigrosin at a concentration of 0.1%. Doubling dilutions of anti-H-2 antisera were made starting at 1:10.

The percentage of dead cells was determined using an inverted microscope (number of stained cells per total number of cells × 100). Test plates were read blindly by two independent observers. The percent cytotoxicity for a given pool of cells is reported as the mean of several values on the plateau portion of the dilution curve. Nonspecific cell death after incubation in C′ alone was ≤5%.

CHIMERISM OF RBC. Blood was collected into acid citrate dextrose. RBC were washed twice in PBS, and made up as a 2% solution in PBS. Serial two-fold dilutions of the specific anti-H-2 alloantisera were made in 1% polyvinylpyrrolidone in PBS with 0.1% bovine serum albumin (freshly added) in a final vol of 0.2 ml in glass tubes (10 x 75). An aliquot of 50 μl of the RBC suspension was added to each tube, and agglutination was read 2 h after incubation at room temperature. Tubes were smoothly stroked across the length of an Rh typing box, and RBC agglutination was scored on a scale of 1+ to 4+ by two independent observers. Data express the maximal hemagglutination that was obtained at the optimal titer of alloantiserum. (B10.A(5R) × A)F_1 anti-B10.A(2R) antiserum was used to detect $H\text{-}2^{b/b}$ and $H\text{-}2^{b/d}$ target cells, and B10 anti-B10.D2 antiserum was used to detect $H\text{-}2^{d/d}$ and $H\text{-}2^{b/d}$ target cells[3] in both the cytotoxicity and hemagglutination assays.

Results

Prolongation of C57BL/Ka Skin Graft Survival on BALB/c Mice Given Different Total Doses of TLI. TLI was administered to three groups of mice

[3] The antisera were kindly supplied by Dr. D. B. Murphy and Dr. H. O. McDevitt, Department of Medicine, Stanford University School of Medicine.

TABLE I

C57BL/Ka Skin Survival (in Days) on BALB/c Mice after Pretreatment with Different Radiation Protocols

Procedure	<20	21-30	31-40	41-50	51-60	61-70	>70	No. of mice	Mean survival time	Range
Control	12							12	10.7	10-13
Splenectomy alone	8							8	11.2	9-15
Subdiaphragmatic irradiation (3,400 rads)	8	6						14	19.9	14-26
TLI 3,400 rads			5	4	5	2		16	49.1	35-67
TLI 3,400 rads + splenectomy				2	3			5	52.0	47-56
TLI 3,400 rads with thymic sheilding	9	1						10	18.0	16-25
TLI 3,400 rads + thymic irradiation			2	6	3		1	12	52.0	24-96

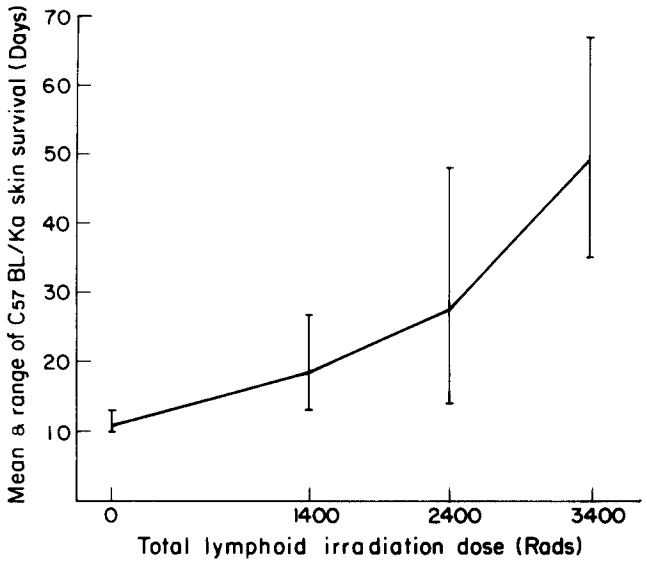

FIG. 2. C57BL/Ka skin allograft survival (mean + range) in BALB/c mice pretreated with different cumulative doses of total lymphoid irradiation.

with cumulative radiation doses of 1,400 (7 mice), 2,400 (15 mice), and 3,400 rads (16 mice), respectively. The mean survival time of C57BL/Ka skin allografts on 12 untreated BALB/c mice was 10.7 days (range, 10-13 days) (Table I). As can be seen in Fig. 2, the effect of TLI on skin allograft survival was dose dependent. Doses of 3,400, 2,400, and 1,400 rads prolonged the mean survival of the skin allografts to 49.1, 27.6, and 18.4 days, respectively. Doses of radiation greater than 3,400 rads were not used in order to avoid radiation damage to visceral organs such as the gastrointestinal tract, kidneys, and liver. The irradiation procedure alone (3,400 rads) did not result in any mortality during an observation period of >250 days.

Prolongation of Skin Allograft Survival on Mice Given Regional Lymphoid Irradiation. The effect of regional lymphoid irradiation was compared to that

of TLI at the same cumulative dose of 3,400 rads. 14 mice were given subdiaphragmatic irradiation using the same apparatus as before except that additional lead was added to shield all areas above the diaphragm. C57BL/Ka skin allograft survival was significantly shorter after regional (19.9 days) as compared to total (49.1 days) lymphoid irradiation (Table I).

Effect of Splenectomy and TLI on Skin Allograft Survival. Five BALB/c mice were splenectomized 3 wk before TLI. The mean survival time of C57BL/Ka skin allografts on the irradiated and splenectomized hosts was 52 days (range, 47–56 days) (Table I). Thus, splenectomy produced no significant further prolongation of allograft survival as compared to TLI alone.

Effect of TLI with Thymic Shielding on Skin Allograft Survival. In order to determine the contribution of thymic irradiation to the prolongation of C57BL/Ka skin allograft survival after TLI, 10 BALB/c mice were given TLI with additional lead shielding placed over the thymus. Table I shows that 9 out of 10 mice in this group rejected their grafts within 20 days. On the other hand, all of 16 mice given TLI without thymic shielding maintained their skin grafts for at least 35 days.

Effect of TLI and Additional Thymic Irradiation on Skin Allograft Survival. The effect of thymic irradiation in addition to TLI was studied in 12 BALB/c mice. A single dose of 2,000 rads was administered to the mediastinum upon completion of TLI. C57BL/Ka skin allografts were transplanted on the following day. The mean survival time of skin allografts was 52 days (range, 24–96 days) (Table I). No thymic tissue could be identified by macroscopic inspection of the mediastinum in three mice that were sacrificed after skin allograft rejection. The results show that additional thymic irradiation did not prolong allograft survival beyond that observed with TLI alone.

Transplantation of Semiallogeneic Bone Marrow to BALB/c Mice Given TLI. Eight BALB/c mice were transfused with 10^7 (BALB/c × C57BL/Ka)F_1 nucleated BM cells, 1 day after completion of TLI (3,400 rads). C57BL/Ka skin was grafted 1 day after BM transfer. Chimerism was documented 180 days after BM transplantation by *H-2* typing of peripheral blood, spleen, and BM lymphocytes, using a dye exclusion microcytotoxic assay (Table II), and by *H-2* typing of RBC using a hemagglutination assay (Table II). Recipient- and donor-type lymphocytes were simultaneously present in the peripheral blood (23 and 77%, respectively), BM (40 and 60%, respectively), and spleen (20 and 80%, respectively). Both recipient- and donor-type RBC were detected in all chimeric mice (Table II).

Transplantation of Allogeneic Bone Marrow to BALB/c Mice Given TLI. A group of 18 BALB/c mice were given TLI (3,400 rads), and an intravenous injection of 10^7 C57BL/Ka BM cells within 24 h after irradiation was completed. C57BL/Ka skin was grafted on the following day. After 6 mo, chimerism of lymphocytes and RBC was assayed in 15 mice that survived. Both donor and recipient lymphocytes and RBC were detected in eight mice with intact skin grafts (Table III). Pooled PBL showed 44% donor-type ($H\text{-}2^b$) lymphocytes, and 52% recipient-type lymphocyte ($H\text{-}2^d$). The lymphoid organs that had been exposed to irradiation contained predominantly donor-type lymphocytes. Thus, 88% of spleen lymphocytes, >95% of lymph node lymphocytes, and 80% of bone

TABLE II
H-2 Typing of Lymphocytes and Red Blood Cells of TLI-Treated BALB/c Mice 6 Mo After Semiallogeneic Bone Marrow Transplantation

Source of Lymphocytes and RBC	Lymphocyte typing		RBC typing	
	Percent kill*		Hemagglutination‡	
	Anti-H-2^d serum	Anti-H-2^b serum	Anti-H-2^d serum	Anti-H-2^b serum
BALB/c (*H-*$2^{d/d}$), peripheral blood§	>95	<5	+3	0
C57BL/Ka (*H-*$2^{b/b}$), peripheral blood§	<5	>95	0	+3
(BALB/c × C57BL/Ka)F_1(*H-*$2^{d/b}$), peripheral blood§	>95	>95	+3	+3
BALB/c + TLI + 10^7 (BALB/c × C57BL/Ka)F_1 BM cells				
Peripheral blood	>95§	77§	+3, +3‖	+1, +1‖
Spleen¶	>98	80		
B.M.¶,**	>98	60		

* Percent kill at the plateau of the dilution curve.
‡ Hemagglutination at the optimal titer of anti H-2 antisera, graded 0 to +3.
§ Tested on pooled blood, seven mice.
‖ Tested on individual blood samples, two mice.
¶ Tested on one individual sample.
** Lymphocytes pooled from exposed and shielded bones (humerus, femur, and tibia).

TABLE III
H-2 Typing of Lymphocytes and RBC of TLI-Treated BALB/c Mice 6 Mo After Allogeneic Bone Marrow Transplantation

Source of lymphocytes and RBC	Lymphocyte typing		RBC typing	
	Percent Kill*		Hemagglutination‡	
	Anti-H-2^d serum	Anti-H-2^b serum	Anti-H-2^d serum	Anti-H-2^b serum
BALB/c (H-$2^{d/d}$), peripheral blood§	>98	<2	+3	0
C57BL/Ka(H-$2^{b/b}$), peripheral blood§	<2	>95	0	+3
BALB/c + TLI + 10^7 C57BL/Ka BM cells				
Peripheral blood	52§	44§	+2 to +3‖	+1 to +3‖
Spleen¶	30	88		
Lymph nodes¶	15	>95		
Bone marrow¶ (shielded: femur and tibia)	65	29		
Bone marrow¶ (exposed: humerus)	26	80		

* Mean of percent kill at the plateau of each dilution curve.
‡ Hemagglutination at the optimal titer of anti-H-2 serum, graded 0 to +3.
§ Tested on pooled blood, seven mice.
‖ Tested on individual samples, seven mice.
¶ Tested on a pool of two mice.

marrow lymphocytes isolated from bones exposed to irradiation (humeri) carried donor-type (*H-*2^b) surface markers (Table III). Conversely, shielded bones (femori and tibiae) contained mostly recipient-type (*H-*2^d) lymphocytes (65% *H-*2^d vs. 29% *H-*2^b). Donor-type lymphocytes were undetectable in blood pooled from

TABLE IV
Fraction of BALB/c Mice with Intact C57BL/Ka Skin Grafts at 250 Days After TLI and Semiallogeneic or Allogeneic Bone Marrow Transplantation

Recipient treatment	No. of mice with intact grafts	No. of mice transplanted
TLI 3,400 rads, i.v. 10^7 (BALB/c × C57BL/Ka)F_1 BM, C57BL/Ka skin graft	7	8
TLI 3,400 rads, i.v. 10^7 C57BL/Ka BM, C57BL/Ka skin graft	8	15*
TLI 3,400 rads, i.v. 10^7 irradiated C57BL/Ka BM, C57BL/Ka skin graft	0	6

* Seven skin grafts were rejected between 34 and 90 days.

the seven mice which rejected their C57BL/Ka skin allografts 34–90 days after transplantation (Table IV).

Lack of Graft vs. Host Disease in Allogeneic Bone Marrow Chimeras. No signs of graft vs. host disease were clinically detectable among BM allotransplant recipients. 15 of 18 mice survived more than 200 days without ruffled fur, hunched back, or diarrhea. Although animals lost up to 25–30% of body weight during irradiation, 1 mo after bone marrow infusion recipients returned to the weight of age-matched controls. Three mice died at 34, 46, and 82 days after BM transplantation. Autopsy of the dead mice revealed: (*a*) interstitial pneumonitis, (*b*) hemorrhagic, fibronecrotic enteritis of the jejunum, and (*c*) vascular damage of small vessels of the liver, and mucosa of the small intestine. There was no splenomegaly. Similar mortality rates and autopsy findings were recorded simultaneously among untreated BALB/c mice in the same mouse room. The cause of death in both cases was not determined.

Specific Transplantation Tolerance in Chimeric Recipients. Seven out of eight BALB/c mice given (BALB/c × C57BL/Ka)F_1 BM cells after TLI treatment maintained C57BL/Ka skin allografts permanently with full hair growth (>250 days). One mouse rejected the skin graft after 95 days (before chimerism was tested) (Table IV). The specificity of tolerance to the allogeneic skin graft was tested by transplanting C3H/He skin to four recipients 200 days after the first C57BL/Ka skin transplantation. Rejection of C3H/He skin grafts occurred after a mean of 8.6 days (range, 7–13 days) while the adjacent C57BL/Ka skin grafts remained intact in all mice.

Eight chimeric BALB/c mice with C57BL/Ka BM cells maintained their C57BL/Ka skin allografts permanently (>250 days). The specificity of tolerance of the chimeras to donor-type alloantigens was tested in the one-way MLR using pooled lymph node cells from two C57BL-Ka-BALB/c chimeras sacrificed after 9 mo. Lymphocytes from the chimeras responded to two types of unrelated stimulator lymphocytes (C3H/He, $H\text{-}2^{k/k}$ and C3H·Q, $H\text{-}2^{q/q}$) but failed to respond to either donor (C57BL/Ka)- or recipient (BALB/c)-type lymphocytes (Table V). The response of normal BALB/c lymphocytes against stimulator cells from the chimeras also documented the presence of allogeneic cells in the chimeric lymphoid organs (Table VI). Tolerance to C57BL/Ka skin allografts could not be established in TLI-treated mice by infusing 10^7 C57BL/Ka BM cells which were irradiated in vitro (3,000 rads) (Table IV).

TABLE V

MLR of Lymph Node Cells from C57BL/Ka-BALB/c Chimeras Against C57BL/Ka, BALB/c, C3H/He, and C3H.Q Stimulator Cells

Responding cells* (5×10^5/culture)	Stimulating cells (1×10^6/culture)	cpm ± SD
C57BL/Ka-BALB/c chimeras	C57BL/Ka-BALB/c chimera	3,817 ± 514
C57BL/Ka-BALB/c chimeras	BALB/c	4,578 ± 448
C57BL/Ka-BALB/c chimeras	C57BL/Ka	5,559 ± 991
C57BL/Ka-BALB/c chimeras	C3H/He	28,424 ± 2,920
C57BL/Ka-BALB/c chimeras	C3H.Q	17,729 ± 3,368

* Pooled lymph node cells from two chimeras obtained 9 mo after marrow transplantation.

TABLE VI

MLR of Normal BALB/c Lymph Node Cells Against C57BL/Ka-BALB/c Chimera, BALB/c, C57BL/Ka, and C3H/He Stimulator Cells

Responder cells (5×10^5/culture)	Stimulator cells (1×10^6/culture)	cpm ± SD
BALB/c	C57BL/Ka-BALB/c chimera*	67,879 ± 3,403
BALB/c	BALB/c	1,715 ± 363
BALB/c	C57BL/Ka	140,749 ± 3,308
BALB/c	C3H/He	95,110 ± 5,143

* Pool of lymph node lymphocytes from two chimeras obtained 9 mo after marrow transplantation.

Number and Function of PBL After TLI. Serial determinations of the absolute numbers of circulating T and B lymphocytes and their in vitro function were carried out after TLI. A group of 12 treated BALB/c mice served as serial blood donors. Values were compared with those of a group of 12 age- and sex-matched untreated BALB/c controls. Pools of blood from both groups were obtained simultaneously at various times after TLI. Mean normal values were calculated by combining data from the serial bleeds of the control group. There were no deaths in the treated group during the 250-day period of observation.

EFFECT OF TLI ON WBC COUNTS. Leukopenia at the completion of TLI was severe, but rapid recovery occurred and the WBC level after 3 wk was in the normal range (Fig. 3 b). However, differential counts revealed an inversion of the lymphocyte/polymorphonuclear ratio (Fig. 3 b). There was an absolute polymorphonuclear leukocytosis of up to 4,500/mm^3 (mean normal counts, 1,690/mm^3), and a concomitant lymphocytopenia.

The lymphocyte/polymorphonuclear ratio increased steadily, and became normal after 3 mo (Fig. 3 b). The total number of WBC was maintained in the normal range from day 20 to day 250 after TLI.

T LYMPHOCYTES. T lymphocytes could not be detected in the peripheral blood during the first 27 days after TLI. Specific killing on day 28 was 14% with the anti-Thy 1.2 antiserum, and the absolute T-cell count was 250 cells-mm^3. During the next 2 wk a steep rise in the absolute number of T lymphocytes was observed (Fig. 3 b). A slow increase in T-cell count continued thereafter, but T lymphocytopenia (>2 standard deviations below the mean of normal controls) persisted for at least 250 days (Fig. 3 b).

B LYMPHOCYTES. Repopulation of Ig-bearing PBL (B cells) began immedi-

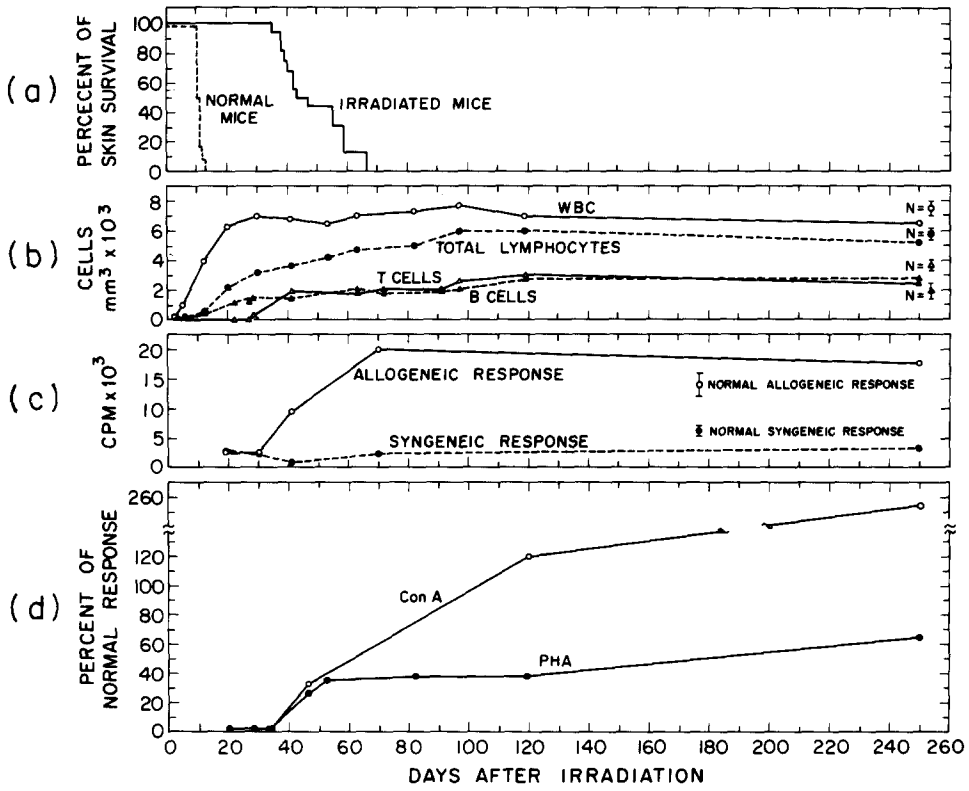

FIG. 3. (a) C57BL/Ka skin allograft survival in 16 BALB/c mice given TLI and in 12 untreated controls. (b) Absolute number of WBC, lymphocytes, T cells (△—△), and B cells (▲—▲) in pooled peripheral blood at different times after TLI. Mean normal values (± standard error) are represented on the right-hand side. (c) One-way mixed lymphocyte reaction of pooled BALB/c PBL against C57BL/Ka lymph node cells at different periods after TLI. Mean (± standard error) syngeneic and allogeneic responses of pooled PBL of untreated BALB/c mice are represented on the right-hand side. (d) Responses of pooled BALB/c PBL to PHA and Con A at different periods after TLI. Responses are expressed as percentages of the normal responses of pooled PBL of untreated BALB/c mice examined concomitantly. Blood samples were obtained from 12 BALB/c mice given TLI (3,400 rads), and from 12 age- and sex-matched control mice.

ately after TLI (Fig. 3b). At day 60 the absolute number of B lymphocytes reached normal levels (1,971 ± 99/mm³). Significant B lymphocytosis (>2 standard deviations above the normal mean) persisted from day 120 (2,700 B lymphocytes/mm³) through day 250 (2,800 B lymphocytes/mm³) after TLI.

PROLIFERATIVE RESPONSE OF PBL TO ALLOGENEIC LYMPHOCYTES (MLR) AFTER TLI. PBL were unable to respond in the one-way MLR against C57BL/Ka lymph node cells for 30 days after TLI (Fig. 3c). A small response was noted at day 41 (9,527 ± 383 cpm). However, complete recovery was noted by day 70 (19,940 ± 436 cpm) (Fig. 3c).

PROLIFERATIVE RESPONSE OF PBL TO PHA AFTER TLI. PBL showed no in vitro response above background to PHA during the first 5 wk after TLI (Fig. 3d). The first response to PHA was noted on day 47, when [³H]thymidine uptake was 24%

of that observed in the normal controls (Fig. 3 d). Diminished PBL responsiveness persisted for 250 days at 40–60% of simultaneous normal control values (Fig. 3 d).

PROLIFERATIVE RESPONSE OF PBL TO CON A AFTER TLI. No response to Con A was observed for 5 wk after TLI (Fig. 3 d). Thereafter, the response rose to 33% of that observed in the matched controls by day 46. The Con A response continued to rise slowly during the remaining period of observation, and supranormal values were measured at 120 (125%) and 250 (255%) days (Fig. 3 d).

Discussion

We have previously shown that total lymphoid irradiation used in the treatment of Hodgkin's disease is a potent immunosuppressive regimen which eliminates the MLR for 2–3 yr, and induces a T lymphocytopenia which persists for at least 10 yr in man (19). Nevertheless, this radiotherapy regimen is associated with few severe side effects other than an increased incidence of herpes zoster (12). Fractionated high dose total lymphoid irradiation (total dose 3,400 rads) can also be administered to mice by appropriate shielding of vital organs such as the lungs and bone marrow (20). After TLI, mice show a long-term T lymphocytopenia, B lymphocytosis, and transient loss of the MLR (20) similar to that observed in man. Our studies of the immune response of treated mice show that the rejection of skin allografts is markedly impaired and that chimerism after infusion of semiallogeneic bone marrow cells occurs for at least 6 mo (21). Animals survive the irradiation procedure well and return to normal weight within a few weeks after treatment (20, 21). The present work extends these observations, and investigates the use of TLI in the induction of specific transplantation tolerance in a completely allogeneic strain combination (C57BL/Ka → BALB/c).

Studies of the relationship between C57BL/Ka skin allograft survival and cumulative dose of TLI given to BALB/c recipients show a sharp falloff in mean survival time between 3,400 (49.1. days) and 1,400 rads (19.9 days). The degree of immunosuppression induced by fractionated irradiation is also dependent upon the extent of the lymphoid tissues included in the radiation fields, since irradiation (3,400 rads) limited to the tissues below the diaphragm prolonged skin graft survival by only 6–7 days. Splenectomy performed before TLI did not result in significant prolongation of graft survival as compared to TLI alone. This finding was of considerable interest, since the majority of patients with Hodgkin's disease treated with TLI were splenectomized before radiotherapy.

In order to determine the contribution of thymic irradiation to the immunosuppression induced by TLI, several mice were given TLI with lead shielding placed over the thymus. C57BL/Ka skin allograft survival was only slightly prolonged [mean survival time (m.s.t.), 18.0 days)] as compared to controls (m.s.t., 10.7 days), and was much shorter than in the group without thymic shielding (m.s.t., 49.1 days). This shows that thymic irradiation plays an important role in the long-term suppression of graft rejection induced by TLI. However, little further prolongation of allograft survival was induced in mice given TLI and an additional single dose of 2,000 rads to the thymus as compared to mice given TLI alone. This suggests that the maximum thymic deficit was

induced by 3,400 rads received by the gland during TLI. Maruyama and Barclay (22) have previously reported that high dose irradiation of the mediastinum can result in a functional thymectomy.

Semiallogeneic (BALB/c × C57BL/Ka)F_1 bone marrow cells transfused intravenously into BALB/c mice treated with TLI survived at least 250 days as judged by stable chimerism of PBL and RBC in seven of eight recipients. The majority of lymphocytes in both the blood and spleen were of donor origin. Stable semiallogeneic bone marrow chimeras showed specific transplantation tolerance. C57BL/Ka skin allografts survived more than 250 days on these recipients, but C3H/He skin grafts transplanted to the same animals at 200 days were rejected within 13 days.

Allogeneic (C57BL/Ka) bone marrow cells and skin allografts also survived at least 250 days in 8 of 15 BALB/c recipients treated with TLI as judged by chimerism of lymphocytes and RBC. Although donor and host contributions to PBL counts were about equal, more than 80% of cells from the spleen, lymph nodes, and BM exposed to irradiation were of donor origin. On the other hand, most of the cells in unexposed BM were of host origin. Surprisingly, allogeneic BM recipients showed no clinical signs (ruffled fur, hunched back, diarrhea, etc.) of graft vs. host disease and continued to gain weight after the cell infusion. The cause of death of 3 of 18 animals after marrow transplantation is difficult to determine, since their autopsy findings were similar to those of untreated BALB/c mice that succumbed in our animal room at the same time. Stable allogeneic chimeras also showed specific tissue tolerance, since lymph node cells obtained from chimeras at 250 days responded to C3H/He cells in the one-way MLR, but did not respond to C57BL/Ka cells.

The increased percentage of stable chimeras induced with semiallogeneic ($^7/_8$) as compared to allogeneic ($^8/_{15}$) bone marrow cells may be due to several mechanisms. One important factor may be the greater immunogenicity of homozygous as compared to heterozygous grafts (23). It is likely that the seven failures observed with allogeneic marrow were due to chronic rejection of these marrow cells, since rejection of skin allografts in these animals occurred after 2–3 mo. A higher percentage of allogeneic bone marrow "takes" may be obtained by increasing the number of infused cells beyond the single dose of 10^7 cells used in the present experiments, since Thomas et al. (24) have shown that the successful engraftment of bone marrow in dogs is directly related to the dose of infused marrow cells.

Serial studies of T lymphocytes in mice given TLI but no allografts showed that T lymphocytopenia persists for at least 250 days. The MLR, PHA, and Con A responses were eliminated at the completion of TLI. However, the MLR returned to normal levels at about day 70. The response to PHA was depressed for at least 250 days, but the Con A response rose to supranormal levels by 120 days.

The alterations in the lymphoid tissues,and immune functions of mice treated with TLI are not yet well understood. However, it is clear that this modality of irradiation produces changes which are more complex than just lymphocyte depletion. Patterns of lymphocyte repopulation after TLI are permanently altered, since abnormalities in the number and function of peripheral blood T

lymphocytes persisit after the absolute lymphocyte count has returned to normal. Similar findings were noted in humans treated with TLI (19).

One hypothesis which is consistent with many of the experimental findings is that TLI not only depletes lymphocytes, but also alters the reticular and endothelial structure of the lymphoid tissues (including the thymus) in such a way that maturation, homing, and cell-cell interactions of T lymphocytes (and perhaps B lymphocytes) may be permanently disturbed. This results in an altered balance in the proportion of T and B lymphocytes, and reduced T-cell subpopulations in the lymphoid tissues (most clearly documented in the peripheral blood). The changes tend to promote the induction of tolerance rather than immunity to antigens introduced during the early recovery phase after TLI. Thus, allogeneic bone marrow cells infused shortly after TLI induce specific transplantation tolerance in host cells. In addition, the donor marrow cells may not mature properly in the irradiated lymphoid tissues and become tolerant to host antigens, thereby eliminating or minimizing the induction of graft vs. host disease. Indirect evidence bearing on these points comes from recent work in our laboratory which shows that two intraperitoneal injections of 40 mg bovine serum albumin in saline induce a state of tolerance (S. Slavin, I. Zan-Bar, and S. Strober, unpublished observations) in BALB/c mice treated with TLI but not in normal controls.

In conclusion, we have developed a technique for the induction of specific tissue transplantation tolerance in adult mice. This technique may have relevance for clinical organ transplantation, since (*a*) the treatment of the recipients (TLI) has been shown to be safe in humans, (*b*) donors and recipients were completely allogeneic, and (*c*) bone marrow and skin allograft survival was permanent (>250 days).

Summary

BALB/c mice were treated with fractionated high dose (3,400 rads) total lymphoid irradiation (TLI), and given semiallogeneic (BALB/c × C57BL/Ka) or allogeneic (C57BL/Ka) bone marrow and/or skin allografts. TLI alone prolonged the mean survival time (m.s.t.) of C57BL/Ka skin grafts to 49.1 days (control, 10.7 days). Shielding of the thymus during TLI produced only a slight increase in graft survival (m.s.t., 19 days). TLI combined with splenectomy was no more effective than TLI alone. Infusion of 10^7 semiallogeneic or allogeneic bone marrow cells after TLI produced stable chimeras in $^7/_8$ and $^8/_{15}$ recipients, respectively. Chimeras were specifically tolerant to donor tissues, since C57BL/Ka skin grafts were accepted for more than 250 days, but third-party (C3H/He) skin grafts were rejected rapidly. In addition, chimeric lymphocytes responded to C3H/He and C3H.Q but not to C57BL/Ka cells in the one-way mixed leukocyte reactions. BALB/c C57BL/Ka chimeras showed no clinical evidence of graft vs. host disease. These findings may have application to clinical organ transplantation, since (*a*) the recipient treatment (TLI) has already been shown to be safe in humans, (*b*) donors and recipients can be completely allogeneic, and (*c*) bone marrow and skin graft survival was permanent (>250 days).

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