Induction of Immunological Tolerance in Full Major and Multiminor Histocompatibility-Disparate Mice Using a Mixed Bone Marrow Transplantation Model

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Abstract. **Bone marrow transplantation (BMT) has been used to induce and maintain immunological tolerance. Such tolerance could facilitate tissue and organ transplantation between the donor and the recipient without need for continuous immune suppression. A protocol employing transplantation of a mixture of T-cell depleted (TCD) syngeneic plus TCD-allogeneic bone marrow cells has been successfully used for induction of transplantation tolerance between mice that differ at components of the major histocompatibility barrier (MHC), or for crossing the xenogenic barrier. We examined the production of specific immunological tolerance using mixed syngeneic plus allogeneic TCD-BMT to cross the entire major plus multiminor histocompatibility barriers. The transplanted mice were repopulated in a stable manner with a mixture of both donor and recipient phenotypes. On histological examination, the mice were reconstituted with hemopoietic and immunocompetent lymphocytes as assayed by their responses to the thymus-dependent cellular antigen, sheep red blood cells (SRBC) in an *in vivo* plaque-forming cell assay. The transplanted mice were found to be stable chimeras and expressed long lasting tolerance of both donor and recipient cells and yet they were fully reactive to third party cells in mixed lymphocyte culture. These results provide evidence that "supportive" or accessory cells in the syngeneic marrow facilitate maturation of donor marrow cells into fully functioning immunocytes in an allogeneic environment crossing the MHC barrier, which represents the greatest known challenge to allogeneic marrow transplantation in mice. The MHC-mismatched mixed allogeneic transplantation method may improve organ engraftment in human recipients of BMT from a partially mismatched donor or from a cadaver donor, and may significantly improve graft acceptance from a fully matched sibling donor.**
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The ultimate aim of transplantation immunology is to achieve successful organ and tissue transplantation without the need for continuous immune suppression (1). Fully allogeneic bone marrow transplantation (BMT), after purging the marrow of destructive T lymphocytes, has been employed as a model system to study T-cell development and differentiation, and to permit analysis of the basis of immunological tolerance across major as well as minor histocompatibility barriers. Fully allogeneic BMT chimeras have been analyzed for tolerance development and immunological capacities (2, 3, 4). These studies showed that mice transplanted from donors which differ from recipients at minor, major, or major plus multiminor histocompatibility barriers could achieve long-term survival. When the mice were transplanted across the fully allogeneic barriers, they were shown to be specifically tolerant of both donor and recipient,

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and yet fully T-cell reactive to third party cells and tissue grafts. However, significant deficiency in primary humoral immune responses (3), and deficient cellular immune responses to intracellular pathogens, e.g., Listeria monocytogenes (5), have made it difficult to maintain such chimeric mice under conventional animal laboratories conditions.

Ildstad, *et al.* (6, 7) showed successful induction of immunological tolerance using a model of mixed host plus donor marrow transplantation after depletion of mature T cells into lethally irradiated, partially MHC-mismatched recipients. The transplanted mice accepted skin grafts of donor origin and showed a long lasting chimeric state.

In the present study, we report the induction and maintenance of specific transplantation tolerance using a mixed transplantation model, which crossed the entire major plus multiminor histocompatibility barriers. Our results show that co-transplantation of syngeneic, along with the allogeneic bone marrow cells significantly improved survival rate and produced chimeras which had impressively normal looking thymus and peripheral lymphoid tissues and lacked immunological deficits of the kinds observed in fully allogeneic BMT chimeras. This stable mixed chimerism was achieved, for the first time, crossing the entire major plus multiminor histocompatibility antigen barrier. Prior investigators, e.g., Ildstad *et al.* (7), crossed the MHC barrier in co-isogeneic strains of mice which did not differ at multihistocompatibility barriers as was tested here.

Lethally irradiated B6 mice were transplanted with a mixture of T-cell depleted (TCD) bone marrow cells from both donor BALB/c and host B6 mice. The mice were followed and analyzed for hematological reconstitution and immunological functions. Our results suggest that syngeneic cells are essential for the full development and functioning of immune cells in allogeneic chimeras.

Materials and Methods

Animals. Inbred, 6–8-week-old female mice of BALB/c (H-2d), C57Bl/6 (H-2b), C3H (H-2k), and DBA/2 (H-2d) strains were purchased from Jackson Laboratories, Bar Harbor, ME. The mice were maintained in the Animal Research Center, All Children's Hospital, St. Petersburg, FL. The mice were housed in sterilized cages on laminar air flow racks and fed regular mouse chow and acidified water.

Cell Preparation. Animals were killed by cervical dislocation. Tibias and femurs were collected and kept on ice in RPMI-1640 medium. Bone marrow was flushed from the bones using RPMI-1640 medium supplemented with 2% fetal bovine serum (FBS) and 1% penicillin-streptomycin using a 5 ml syringe with 25 gauge needle. Spleens were collected and disrupted in RPMI-1640 medium (supplemented with 2% FBS, 1% penicillin-streptomycin, 1% HEPES buffer, and 1% glutamine) by pressing spleen fragments between two sterile frosted glass slides, and the cells were carefully resuspended into a fully dispersed suspension of single cells.

Depletion of Mature T Lymphocytes. Bone marrow cells from donor and host strain were incubated with anti Thy-1.2 monoclonal antibody (1 μg/ 5×10^6 cells, Becton Dickinson, Mountain View, CA) for 30 min at 4°C. The cells were washed twice in RPMI-1640 medium supplemented with 1% penicillin-streptomycin by centrifugation for 5 min at 1600 × g, then adjusted to 10×10^6 cells/ml. The cells were then incubated with low toxicity rabbit complement (Pelfreeze, Brown Deer, WI) for 45 min at 37°C and then washed twice in RPMI-1640 medium. Cells were counted using a hemocytometer and cell viability evaluated by trypan blue dye exclusion.

Protocol for Bone Marrow Transplantation. B6 recipients were given 950 r of total body irradiation from a ^{137}Cs gamma source 20 hr before transplantation. On the day of transplantation, recipients were injected in a lateral tail vein with an appropriate number of cells of either: (i) syngeneic bone marrow, each mouse was given a dose of $10–20 \times 10^6$ cells. (ii) allogeneic TCD-bone marrow, each mouse was injected with $20–25 \times 10^6$ cells of donor origin. (iii) mixed syngeneic plus allogeneic marrow, each host was given 5×10^6 TCD-bone marrow of syngeneic origin plus 15×10^6 TCD-bone marrow of allogeneic strain origin.

Post Transplantation Analysis. The mice were observed for survival in a clean conventional environment (laminar air flow racks). One hundred days post-transplantation, the reconstituted mice were analyzed for the following parameters:

Analysis of Chimerism. Spleen cells from control and transplanted animals were analyzed for chimerism by immunostaining with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies specific for H-2b, H-2d, and H-2k MHC antigens (Pharmingen, San Diego, CA). 100 μl of spleen cells at a concentration of 10×10^6/ml in phosphate buffered saline (PBS) supplemented with 2% FBS, after lysis of the red blood cells, were incubated with the different monoclonal antibodies for 30 min at 4°C. The cells were washed once, resuspended in PBS and the fluorescence was detected by flow cytometric analysis (EPICS Elite, Coulter).

***In Vivo* Anti–Sheep Red Blood Cells (SRBC) Plaque-Forming Cell (PFC) Assay.** To test for primary antibody production, each treated and control mouse was injected with 0.5 ml of 2% SRBC suspension in complete Hanks balanced salt solution (HBSS) by intraperitoneal injection. Five days later the mice

were killed and spleen cells were adjusted to a concentration of 5×10^6 cell/ml in HBSS. SRBC were washed twice, white blood cells carefully removed from atop the SRBC and the cell pellet was suspended in HBSS at a concentration of 30%. In a small glass tube (in triplicates or duplicates), a mixture of 500 μl of 5% agarose (Sigma) solution, 100 μl of spleen cell suspension, and 50 μl of SRBC solution was quickly vortexed and poured onto an agarose coated microslide. Dried slides were carefully inverted in a tray and incubated with guinea pig complement (Cappel, West Chester, PA) diluted 1:30 in HBSS at 37°C. After 3 hr, hemolytic plaques were counted and expressed as number of plaques per 5×10^5 cells.

Mixed Lymphocytic Culture. Spleen cells (1×10^5) from recipients were cultured with 1×10^5 irradiated (3000 r) syngeneic, allogeneic, or third party spleen cells in a U-bottom 96 well tissue culture plate in 0.2 ml of RPMI-1640 medium supplemented with 10% FBS, 1% glutamine, 1% penicillin-streptomycin, 1% HEPES buffer and 50 μ*M* 2-mercaptoethanol. The cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for four days, then pulsed with 1 μCi of labeled ^{3}H-thymidine. The cells were incubated for one more day and thymidine incorporation into DNA was measured using a liquid scintillation counter. Results are presented as the stimulation index value which represents the mean counts per minute (cpm) of experimental cultures divided by the mean cpm of cultures of recipient cells with autologous cells.

Histological Analysis. Histological sections were prepared from spleens and thymuses of the transplanted as well as age and sex-matched control nontransplanted mice. Histological sections were reviewed mainly for the general architecture of the spleen and of the thymus. The spleen was analyzed for the presence and extent of lymphoid follicles and, as a site of hemopoiesis, was also observed for reconstitution with myeloid, erythroid, and megakaryocytic elements in the red pulp. The thymus was analyzed with particular attention to the lymphoid cortex and a distinct cortico-medullary junction and cortico-medullary ratio. All observations were made on routine Hematoxylin and Eosin-stained sections 100 days post transplantation unless otherwise indicated.

Statistical Analysis. Results of observations obtained from multiple experiments were reported as the mean ± SEM (standard error of the mean). The *P* value was calculated using generalized Wilcoxon test. A *P* value of <0.05 was taken to be significant.

Results

Long-Term Survival of B6 Mice Transplanted with a Mixture of Syngeneic Plus Allogeneic T-Cell Depleted-Bone Marrow Cells. Figure 1 compares survival data for B6 mice reconstituted with different preparations of marrow cells 100 days post transplantation. Control irradiated mice that were not given bone marrow died within 15 days. The groups compared for survival after transplantation with TCD-bone marrow cells were reconstituted as follows: (i) 10×10^6 syngeneic bone marrow cells (B6 → B6), (ii) $15–20 \times 10^6$ allogeneic TCD-bone marrow cells (BALB/c → B6), (iii) mixed TCD-syngeneic (5×10^6) plus TCD-allogeneic (15×10^6) marrow cells (BALB/c + B6 → B6). Survival of mice in the three groups was in a similar range, however, the mixed transplantation protocol achieved a significantly better survival rate ($P < 0.05$) under the conditions of this study. Mice of all the transplanted groups appeared healthy, gained weight, and did not show any evidence of wasting, diarrhea, or any other signs associated with graft vs host disease or gross immunodeficiency.

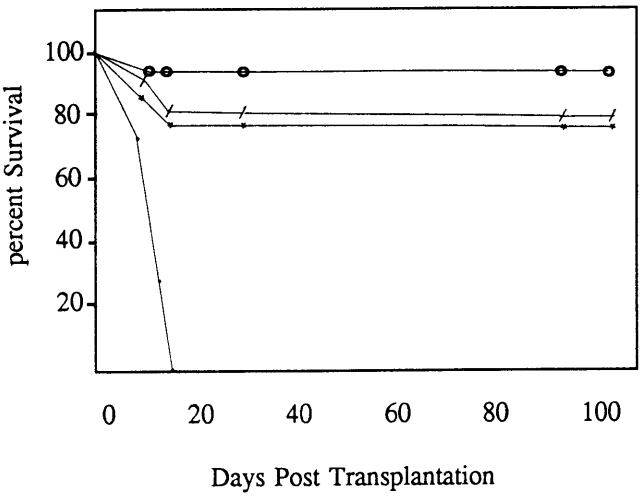

Figure 1. Survival of B6 mice transplanted with T-cell depleted bone marrow. ○, Mixed chimeras (BALB/c + B6 → B6, $n = 45$) achieved significantly better survival ($P < 0.05$) than the other transplanted groups; *, fully allogeneic chimeras (BALB/c → B6, $n = 40$); /, transplantation of syngeneic marrow cells (B6 → B6, $n = 20$); and ▪, irradiation control ($n = 4$).

Flow Cytometric Analysis of the Chimeric State of B6 Mice Transplanted with TCD-Bone Marrow Cells from BALB/c Mice. As shown in Figure 2, spleens from B6 mice transplanted with allogeneic (BALB/c) TCD-bone marrow cells were repopulated almost completely with cells of donor origin (H-2d). Mice transplanted with a mixture of syngeneic and allogeneic TCD-bone marrow cells expressed a mixed phenotype (H-2b and H-2d); the donor phenotype however (H-2d) was generally dominant. Control mice transplanted with syngeneic marrow cells always expressed an (H-2b) phenotype (data not shown) as in control nontransplanted B6 mice.

Vigorous Primary Antibody Response in B6 Mice Transplanted with Mixed Allogeneic TCD-Bone Marrow Cells. B6 mice transplanted with TCD-

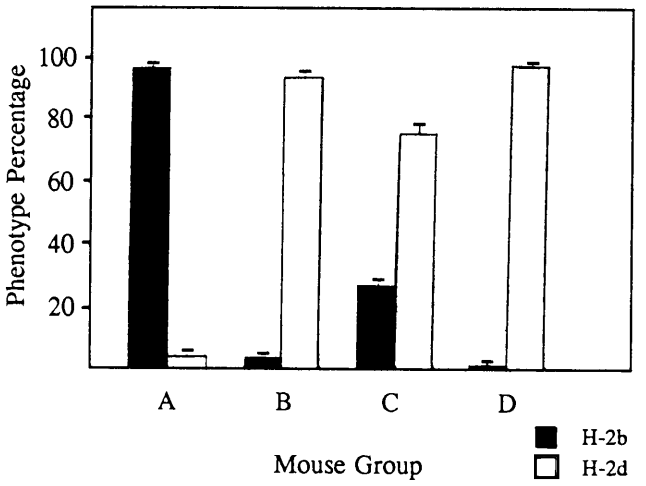

Figure 2. Phenotype of spleen cells in B6 mice transplanted with T-cell depleted marrow cells was determined 100 days post transplantation using flowcytometer analysis. A, normal B6 mice; B, fully allogeneic chimeras (BALB/c → B6); C, mixed chimeras (BALB/c + B6 → B6); and D, normal BALB/c mice. Bars represent mean ± SEM (n = 3–4).

bone marrow cells were tested for their ability to mount a primary antibody response against a cellular antigen, SRBC in a plaque-forming cell assay. Mice transplanted with syngeneic marrow cells produced a vigorous primary antibody response that was slightly higher than was observed in control nontransplanted mice (Fig. 3, C vs B). However, inhibition of primary antibody formation was observed in mice that had been transplanted with an allogeneic bone marrow (Fig. 3, D), while animals reconstituted with a mixture of syngeneic plus allogeneic cells exhibited a prominent IgM primary response that was comparable to

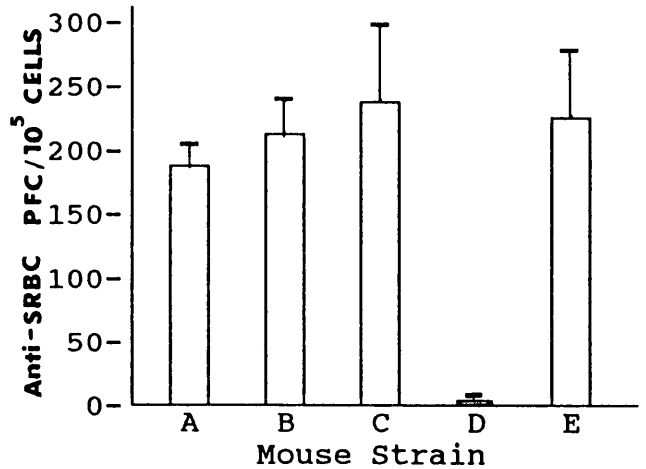

Figure 3. Primary anti-SRBC plaque-forming cell (PFC) assay in B6 mice transplanted with TCD-bone marrow cells. A, BALB/c mice; B, B6 mice; C, syngeneic BMT (B6 → B6); D, TCD-allogeneic BMT (BALB/c → B6); and E, mixed TCD-syngeneic plus TCD-allogeneic BMT (BALB/c + B6 → B6). Bars represent mean ± SEM (n = 3–4). Results represent multiple experiments.

that of mice reconstituted with syngeneic marrow (Fig. 3, E vs C).

Tolerance and Alloreactivity of B6 Mice Transplanted with TCD-Bone Marrow Cells. Spleen cells from transplanted B6 mice were tested for the capability to proliferate in response to donor strain cells in a mixed lymphocyte culture. As shown in Table I, cells from mice reconstituted with syngeneic marrow cells (B6 → B6) were tolerant of donor (B6) cells but vigorously alloreactive to other strain cells (BALB/c and C3H). Spleen cells of allogeneic chimeras (BALB/c → B6) were tolerant of both donor (BALB/c) and recipient (B6) cells but vigorously alloreactive to third party cells (C3H). Cells from mixed chimeras (BALB/c + B6 → B6) were also tolerant of both donor strains (BALB/c and B6), and also fully reactive to third party cells.

Histological Examination of Spleens and Thymuses from B6 Mice Transplanted with TCD-Bone Marrow Cells. Spleen sections from B6 mice transplanted with TCD-bone marrow cells were examined histologically for hemopoietic and lymphoid reconstitution. As shown in Figure 4, apparently complete reconstitution of the lymphoid architectural pattern was observed in the mice of the group that had been reconstituted with a mixture of syngeneic plus allogeneic TCD-bone marrow cells. The architecture of the spleen and its red and white pulp was preserved. Hemopoietic cells including megakaryocytes, myelocytes and macrophages, and lymphoid cells were detected in a distribution comparable to that present in normal nontransplanted mice (Fig. 4, D vs A). In mice transplanted with only allogeneic marrow without the syngeneic component (C), megakaryocytes and normoblasts were extremely few in number in red pulp of the spleen. Irradiated mice that were not reconstituted with bone marrow cells (E) showed atrophic spleens, atrophic remnants of follicles, and also exhibited accumulations of hemosiderin due to lysis of red blood cells. In these irradiated mice, no evidence of erythropoiesis or lymphopoiesis was observed in the spleen, and plasma cells were absent.

Thymus sections from the same groups of mice were examined for lymphoid reconstitution. As shown in Figure 5, in mixed allogeneic-reconstituted mice (D), an essentially normal thymic architecture with lymphoid development in thymic cortex and a fainter staining medulla was observed. The thymic medulla showed reactive hyperplasia in mice transplanted with syngeneic (B) or a mixture of syngeneic plus allogeneic (D) TCD-bone marrow cells. On the contrary, in mice that were given allogeneic TCD-BMT across the MHC (C), thymuses were somewhat involuted and were lacking a distinct corticomedullary demarcation. In control irradiated nontransplanted animals (not

Table I. Mixed Lymphocyte Culture (MLC) Response in B6 Recipients 100 Days Post Transplantation of T Cell Depleted[a] Bone Marrow Cells

Responder	# of mice	Stimulator			
		BALB/c	B6	C3H	Self
B6*	3	5.6† ± 3.2	1.0 ± 0.0	5.4 ± 4.6	—
BALB/c*	2	1.0 ± 0.0	10.1 ± 1.0	5.5 ± 0.4	—
C3H*	3	3.1 ± 1.9	3.8 ± 0.6	1.0 ± 0.0	—
B6 → B6	2	5.6 ± 1.4	1.1 ± 0.1	5.2 ± 0.1	1.0 ± 0.0
BALB/c → B6	3	0.5 ± 0.4	0.5 ± 0.1	3.0 ± 1.5	1.0 ± 0.0
BALB/c + B6 → B6	5	1.3 ± 0.4	1.0 ± 0.2	3.0 ± 1.8	1.0 ± 0.0

[a] Spleen cells from transplanted or nontransplanted* mice (responder) were tested for proliferation by incubation with irradiated (stimulator) spleen cells as described in the Materials and Methods section.
† Mean of stimulation index = mean cpm of experimental culture/mean cpm of culture with autologous cells ± SEM.

shown), the thymus, particularly the cortex, was dramatically atrophic and exhibited few epithelial cells and no detectable Hassall's corpuscles.

Discussion

Bone marrow transplantation has been employed to induce transplantation tolerance across strain and species barriers (3, 6, 8–11). In prior studies, Ildstad *et al.* (6, 7) reported induction of immunological tolerance using a mixture of syngeneic plus allogeneic TCD-bone marrow cells between congeneic strains of mice (B10 and B10-D2). In the present study, we report the successful application of this method for induction and maintenance of transplantation tolerance crossing the entire major plus multiminor histocompatibility antigen barriers. Our mixed chimeras were superior to fully allogeneic chimeras when comparing histological criteria and immune functions. Fully allogeneic chimeras, which were reconstituted mostly with cells of MHC-mismatched donors, exhibited serious deficiency in the primary humoral immune responses and susceptibility to certain intracellular bacterial infections indicating deficiency also of cellular immune functions (2, 3, 5). We applied a mixed transplantation protocol in an effort to obviate these immune deficiencies in mice transplanted with fully MHC plus multiminor histocompatibility-divergent donor cells (BALB/c + B6 → B6) (12, 13). Mixed allogeneic chimeras achieved an excellent survival in a clean conventional environment and produced stable and long-lasting mixed chimeras that contained more donor than recipient type cells. When these mice were examined 200 days post transplantation, the percentage of donor type cells in the spleen was approximately equal to the percentage of host type cells (data not shown). Thus, these chimeras were both highly stable and impressively reconstituted.

The primary antibody responses to a thymus-dependent cellular antigen, SRBC was vigorous in mixed chimeras and comparable to responses obtained following syngeneic BMT. These responses were grossly deficient in fully allogeneic BMT chimeras. Mixed chimeras expressed intact cellular immune responses and specific transplantation tolerance since their spleen cells were tolerant of both donor and recipient yet vigorously reactive to third party cells as tested in an MLC. Healthy mixed chimeras were also produced in a reverse order of transplantation of these same mouse strains (BALB/c + B6 → BALB/c, results are not shown).

On histological examination of spleen and thymus sections, fully allogeneic chimeras differed from mixed syngeneic plus allogeneic chimeras. In the latter, mice were repopulated with hemopoietic and lymphoid elements, appeared as normal as syngeneic marrow transplants and quite comparable to sections from normal B6 mice. On examining spleen sections from several fully allogeneic chimeras, we failed to find megakaryocytes, which suggests that the fully allogeneic barrier might interfere with full hemopoietic reconstitution. However, no evidence of bleeding or other indication of hemopoietic failure was observed in these chimeras. Detailed analysis of hemopoiesis and quantitative deficits of platelets must await further investigations. It is possible that fully allogeneic barrier presents problems in establishing regular hemopoiesis as compared to the syngeneic transplants or mixed host plus donor marrow transplants. Thymus sections from fully allogeneic chimeras showed evidence of medullary hypoplasia indicating a possible quantitative deficiency in thymocyte development and/or function. These results are in accordance with observations previously reported (5) indicating cellular immune deficiency to intracellular pathogens, although no deficiency in cellular proliferation to foreign cellular antigen was observed by others (3), and as was also noted in our study findings.

These results indicate the necessity of syngeneic elements for the production of healthy chimeras which have normal hemopoiesis and lymphoid development

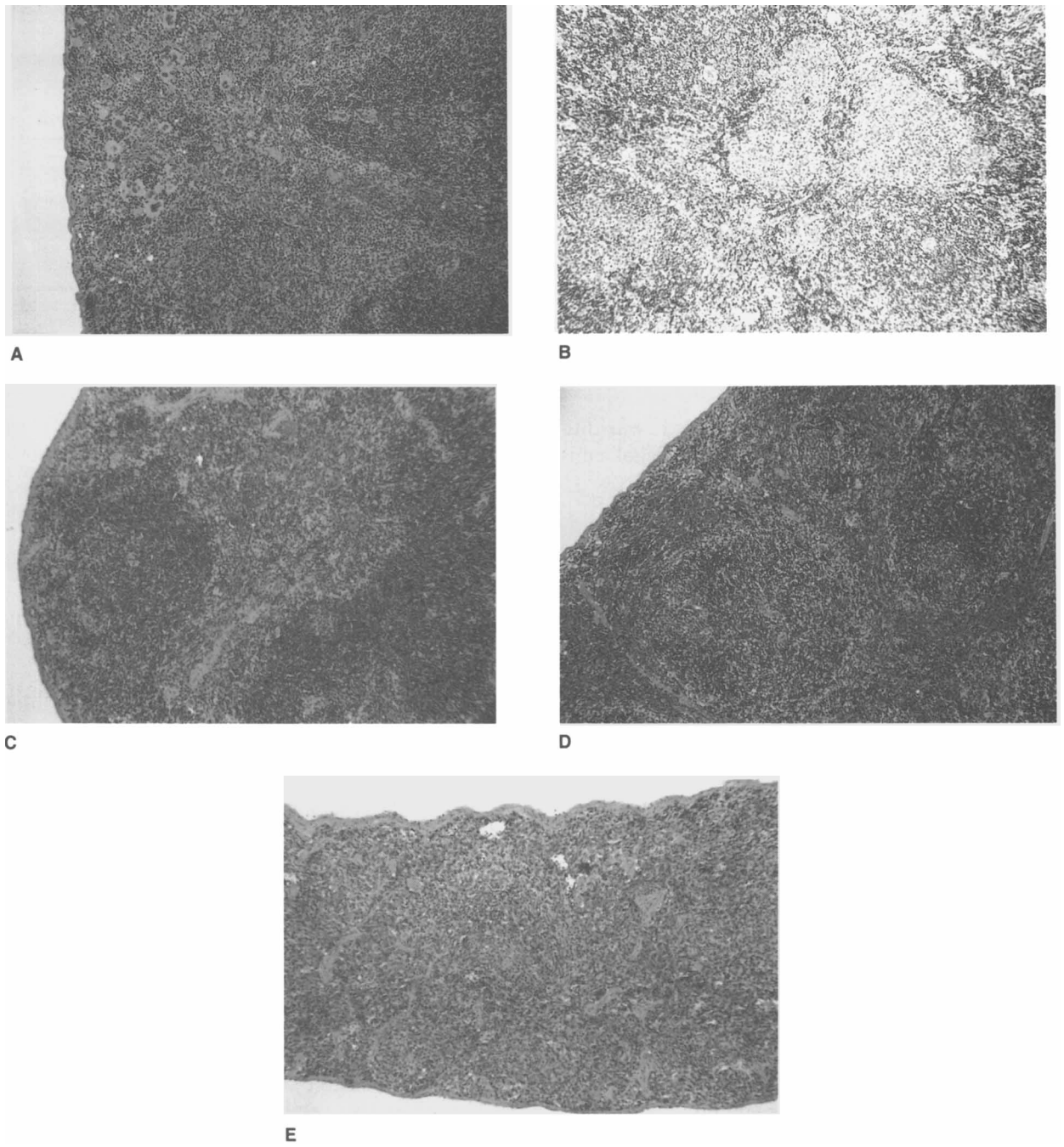

Figure 4. Histological sections of spleens from B6 mice transplanted with bone marrow cells. Hematoxylin and Eosin sections (×100) were examined 100 days post transplantation except in group E that was examined 10 days post transplantation. A, Normal B6 (nontransplanted); B, syngeneic BMT (B6 → B6); C, TCD-allogeneic BMT (BALB/c → B6); D, mixed TCD-syngeneic plus TCD-allogeneic BMT (BALB/c + B6 → B6); E, irradiation control (B6, nontransplanted).

in a conventional environment, especially when the MHC antigen barrier must be crossed. Onoé *et al.* (4) reported that adoptive transfer of B-cells from chimeras plus a "non–T-cell preparation" from an MHC-incompatible donor into an irradiated donor crossed with host F1 recipient resulted in restoration of the defective primary antibody responses. Recent studies by Starzl *et al.* (14, 15) report the persistence of a mixed chimerism of dendritic cells in the peripheral tissues and organs in recipients of successful kidney, liver, and heart allografts. These dendritic cells persist for many years after engraftment without evidence of rejection and apparently produced what the authors conclude is a thymus-independent form of tolerance. Dendritic cells may play an important role in the production of healthy, stable chimeras, if transplanted along with allogeneic bone marrow cells. Experiments by Fan *et al.* (16) showed that injected allogeneic bone marrow cells can migrate and proliferate by interaction with the stroma of an allogeneic piece of bone trans-

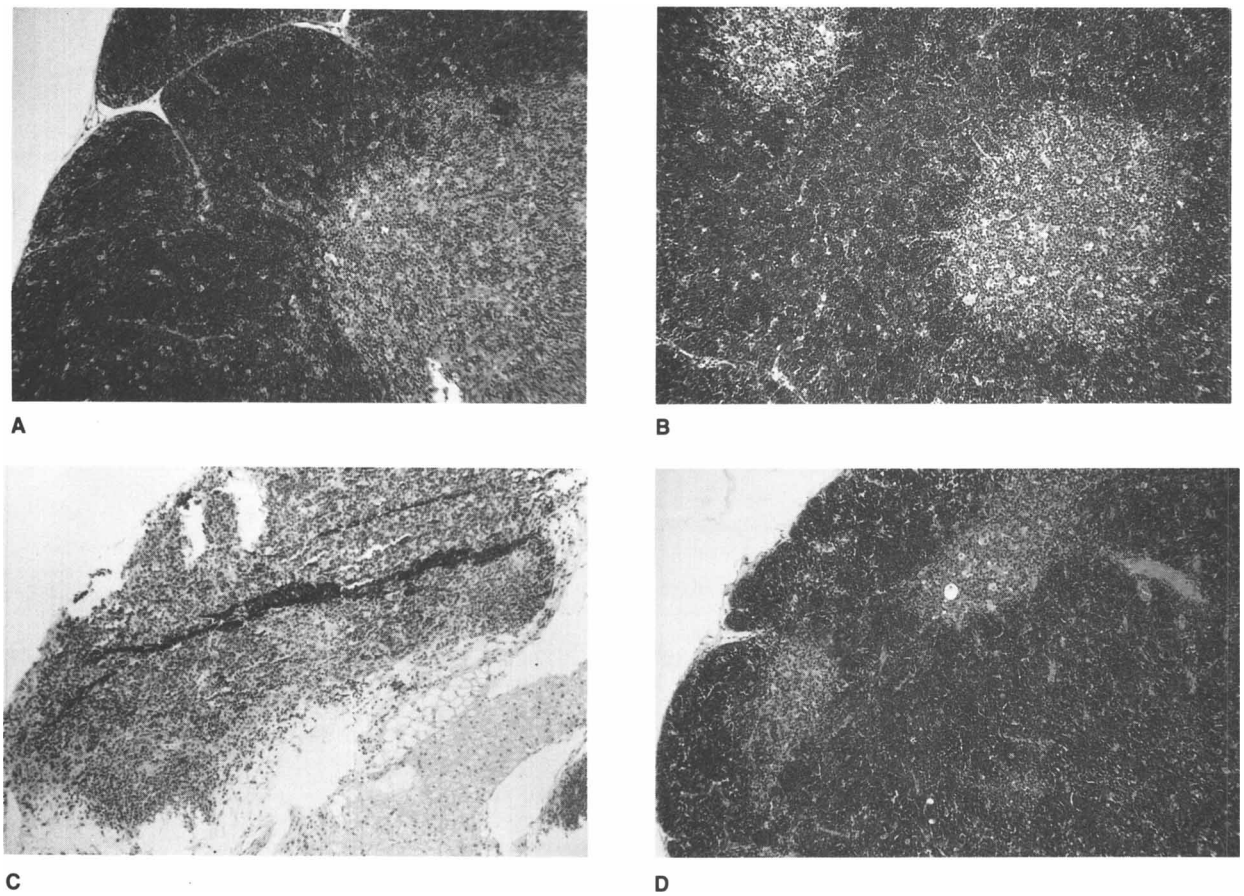

Figure 5. H & E histological sections (×100) of thymuses of B6 mice transplanted with bone marrow cells. A, Normal nontransplanted B6; B, syngeneic BMT (B6 → B6); C, allogeneic TCD-BMT (BALB/c → B6); D, mixed TCD-syngeneic plus TCD-allogeneic BMT (BALB/c + B6 → B6).

planted under the kidney capsule of the recipient. Recent studies by Perkins *et al.* (17, 18) have shown that stromal cells of the bone marrow microenvironment consist predominantly of an endothelial-like cell population that expresses collagenase IV and laminin. These cells are not transplanted in the usual bone marrow graft, hence, donor hemopoiesis in allogeneic chimeras must be supported by host stroma. Our results showing successful alloengraftment and maturation of bone marrow cells into functioning immune cells in mixed chimers suggest that transplantable bone marrow-derived cells, such as macrophages and dendritic cells, and not endothelial cells, may become the supportive cells essential for bone marrow engraftment and full development in an allogeneic microenvironment.

Soluble mediators such as interleukin-2 (IL-2) produced a limited effect on alloengraftment which has been demonstrated in some strains but not others (19). In other experiments, administration of IL-2 restored the *in vitro,* but not the *in vivo* anti-SRBC plaque-forming cell response (20). Current studies in our laboratory are being pursued to further characterize the cellular/environmental factors necessary to correct cellular and humoral immune deficits in fully allogeneic chimeras. Achieving a fully functioning immune system in fully allogeneic chimeras could have a significant impact on bone marrow transplantation by increasing the immunological competence of the recipients without compromising graft acceptance. If so, the model of mixed syngeneic plus allogeneic chimerism shown herein to permit the development of long-term stable mixed chimerism, strong cell mediated immunities as well as vigorous primary and secondary antibody responses must be considered as an important approach to tolerance development and treatment for many human as well as experimental diseases.

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