Brief Definitive Report

CELLULAR BASIS OF GRAFT VERSUS HOST TOLERANCE IN CHIMERAS PREPARED WITH TOTAL LYMPHOID IRRADIATION*

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High-dose fractionated total lymphoid irradiation (TLI), a radiotherapy technique used for human lymphomas, allows for the engraftment of completely allogeneic bone marrow (BM) without clinical graft vs. host disease (GVHD) in adult inbred rodents and outbred dogs (1–5). TLI-treated BALB/c (H-2^d) mice given 30×10^6 C57BL/Ka (H-2^b) BM cells developed stable mixed chimerism in the blood and lymphoid tissues and specific tolerance to C57BL/Ka skin grafts. Donor-type (H-2^b) cells comprised 60–90% of peripheral blood lymphocytes (PBL) and ~90% of splenocytes in the chimeras (1). C57BL/Ka → BALB/c chimeras prepared with TLI developed no clinical signs of GVHD and routinely survived >100 d. In contrast, BALB/c mice given lethal whole-body irradiation and 30×10^6 C57BL/Ka BM cells developed typical GVHD resulting in 60% mortality at 15 d and 95% mortality at 60 d after BM transfer (2).

Previous studies (6) showed that the injection of C57BL/Ka → BALB/c chimeric spleen cells into lightly irradiated (550 rad) BALB/c mice did not lead to GVHD even though 90% of the cells were of donor (C57BL/Ka) origin. These sublethally irradiated recipients, in turn, became stable mixed chimeras and accepted C57BL/Ka skin grafts (6). Injection of chimeric cells into third-party hosts was not attempted. The absence of GVHD in the mature TLI chimeras and in irradiated BALB/c recipients of chimeric spleen cells had a number of possible explanations. These included clonal deletion, active suppression by donor and/or host cells, blocking by serum factors, or general immunodeficiency of donor-type cells maturing in a completely allogeneic thymic microenvironment. A possible role for host suppressor cells was suggested by studies in which spleen cells from BALB/c mice given TLI without BM were found to exert a potent suppressor effect on mixed leukocyte reactions (MLR) regardless of the identity of the responder and stimulator cells (7). When injected along with C57BL/Ka BM, these suppressor cells protected the majority of lethally irradiated BALB/c mice from acute GVHD.[1]

In this work, we used a GVHD mortality assay to study the mechanism of the graft vs. host tolerance in the completely allogeneic mixed chimeras prepared with TLI. The results indicate that donor-type (C57BL/Ka) lymphocytes in long-term TLI

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chimeras are specifically tolerant to the tissues of the host. Thus, lack of GVHD is not dependent upon the continued presence of suppressor cells of host (BALB/c) origin or on chronic immunodeficiency of the donor-type cells.

Materials and Methods

Chimeras. BALB/c (H-2^d) mice, 4–6-mo old, were given TLI in 17 fractions of 200 rad each as described previously (1). 1 d later, BM cells (30×10^6) from C57BL/Ka (H-2^b) donors were infused via the lateral tail vein. Chimerism of the PBL of individual BM recipients was assayed at least 40 d after transplantation by a complement (C′)-dependent microcytotoxicity assay using BALB/c anti-C57BL/Ka antiserum (1).

Adoptive Transfer Recipients. Adult (3- to 6-mo old) male and female BALB/c and C3H (H-2^k) mice were used as whole-body irradiated (WBI) recipients of chimeric and normal spleen cells in GVHD mortality experiments. WBI was delivered using a 250-kV, 15-mA Philips unit (Philips Electronic Instruments, Inc., Mahwah, N. J.) at a dose rate of 93 rad/min. Neomycin sulfate (500 μg/ml) and polymyxin B (100 μg/ml) were added to drinking water before WBI and continued indefinitely.

Isolation of Chimeric Spleen Cells of Donor (C57BL/Ka) Origin. Four to six C57BL/Ka → BALB/c chimeras (>100 d after transplantation) were used for each experiment. Single-cell suspensions were prepared in cold RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.). The cells were spun at 250 *g* and resuspended at 10×10^6/ml in RPMI-1640 medium that contained 0.3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). C57BL/Ka anti-BALB/c alloantiserum was added to a final concentration of 1:50 for 30 min at 37°C. After spinning at 250 *g* for 10 min at room temperature, the unbound antiserum was removed. The cells were resuspended (20×10^6/ml) in rabbit C′ (1:8) (Lo-Tox; Cedarlane Medical Laboratories Ltd., Ontario, Canada) at 37°C for 45 min and then spun for 10 min at 250 *g*. The cells were washed two times in fresh medium, counted, and adjusted to the appropriate concentration in RPMI-1640. In preliminary experiments with antiserum concentrations of 1:100 or greater, this procedure resulted in 90–100% cytotoxicity for BALB/c spleen cells and <5% cytotoxicity for normal C57BL/Ka spleen cells.

Results and Discussion

BM Transplantation without GVHD in Mice after TLI. More than 90% of BALB/c mice given TLI followed 1 d later by C57BL/Ka BM cells (25×10^6) developed stable mixed chimerism. Cytotoxicity assays were performed on PBL from individual mice at least 40 d after BM transfer. PBL from chimeric recipients contained 50–90% donor-type (H-2^b) cells. C57BL/Ka skin grafts survived indefinitely on chimeric animals. None of the marrow recipients showed clinical signs of GVHD. Spleen cells obtained from chimeras >100 d after BM transplantation were used for all cell transfer experiments.

Specific Absence of GVHD in Mice Given Sublethal WBI and Chimeric Spleen Cells. 9 of 10 BALB/c mice given 450 rad WBI followed 24 h later by an intravenous infusion of chimeric spleen cells (25×10^6–50×10^6) survived >45 d with no signs of GHVD (Table I). BALB/c recipients given 450 rad and normal C57BL/Ka spleen cells (25×10^6) developed characteristic signs of acute GVHD (diarrhea, weight loss). Only 1 of 10 survived >15 d. All of 9 BALB/c mice given 550 rad WBI and chimeric spleen cells (25×10^6) survived >45 d, whereas none of 22 similarly irradiated recipients of normal C57BL/Ka spleen cells (25×10^6) survived >15 d. Donor-type (H-2^b) cells were present in the PBL of two of nine recipients of chimeric spleen cells when tested at least 40 d after transfer.

To assess the specificity of graft vs. host tolerance in the C57BL/Ka → BALB/c

TABLE I

Survival of Mice Given Sublethal WBI and Chimeric Spleen Cells

Strain	Recipient treatment	Spleen cell source	Fraction surviving			No. chimeras/No. tested*
			15 d	30 d	45 d	
	rad					
BALB/c	450	None	10/10	9/10	9/10	
BALB/c	450	C57BL/Ka (25×10^6)	1/10	0/10	—	NT
BALB/c	450	Chimera ($25–50 \times 10^6$)	10/10	10/10	9/10	0/9
BALB/c	500–550	None	37/47	37/47	37/47	
BALB/c	500–550	C57BL/Ka (25×10^6)	0/22	—	—	NT
BALB/c	500–550	Chimera (25×10^6)	9/9	9/9	9/9	2/9
C3H	650	None	40/44	39/44	38/44	
C3H	650	C57BL/Ka (25×10^6)	0/26	—	—	NT
C3H	650	Chimera (25×10^6)	3/5	0/5	—	NT

NT, not tested.

* Fraction of animals with ≥16% donor-type (C57BL/Ka) PBL at least 40 d after cell transfer.

chimeras, third-party mice of the C3H strain (H-2^k) were treated with sublethal WBI (650 rad) and given 25×10^6 chimeric spleen cells intravenously. C3H recipients were treated with a higher radiation dose to achieve a similar radiation mortality to that observed with BALB/c mice given 500–550 rad WBI (Table I). None of five survived >30 d after cell transfer. 38 of 44 mice given radiation alone survived >45 d, but none of 26 given WBI and normal C57BL/Ka spleen cells survived >15 d. These experiments suggested, but did not prove, that the donor-type cells in chimeric spleen were both immunocompetent and specifically tolerant to host tissues. The possibility remained that the GVHD observed in third-party mice was mediated by cells of host origin (~20% of the chimeric spleen cells), and that lack of GVHD in BALB/c recipients was mediated by suppressor cells of host origin. These alternative explanations pointed to the need to assess the graft vs. host reactivity of an isolated population of chimeric donor-types cells.

Specific Absence of GVHD Potential of Donor-Type (H-2^b) Chimera Spleen Cells. We isolated donor-type (C57BL/Ka) spleen cells from C57BL/Ka → BALB/c chimeras >100 d after BM transplantation. Pooled spleen cells (~80% C57BL/Ka) were treated in vitro with C57BL/Ka anti-BALB/c alloantiserum (anti-BALB/c) and rabbit C′ to eliminate cells of host (BALB/c) origin. The remaining cells were washed, and aliquots that contained 25×10^6 viable cells were injected intravenously into sublethally irradiated BALB/c and third-party (C3H) hosts (Table II). Data in Table II was pooled from at least two experiments of each type. 17 of 20 BALB/c recipients of chimeric donor-type spleen cells survived in apparent good health for >60 d after cell transfer. In contrast, irradiated C3H recipients of the same cells developed a severe wasting syndrome. 2 of 12 survived >15 days, and none >30 d. 85% of mice given radiation alone survived >60 d. The mortality of C3H mice given WBI and normal C57BL/Ka spleen cells was 100% at 15 d. The results show that cells of donor origin in the chimeras have lost their reactivity to the tissues of the host strain even when removed from the chimeric environment, but can mediate GVHD against a third-party strain.

TABLE II

Survival and Chimerism of Mice Given WBI and Chimeric Spleen Cells

Strain	Recipient treatment	Spleen cell source	Fraction surviving				No. chimeras/No. tested*
			15	30	45	60	
	rad						
BALB/c	500–550	None	37/47	37/47	37/47	37/47	
BALB/c	500–550	C57BL/Ka (25×10^6)	0/22	—	—	—	NT
BALB/c	500–550	C57BL/Ka (20×10^6) + BALB/c (20×10^6)	11/14	11/14	11/14	11/14	0/6
BALB/c	500–550	C57BL/Ka (20×10^6) + BALB/c (20×10^6 anti-BALB/c treated)‡	0/12	—	—	—	NT
BALB/c	500–550	Chimera (25×10^6 anti-BALB/c treated)‡	17/20	17/20	17/20	17/20	9/20§
C3H	650	None	40/44	39/44	38/44	38/44	
C3H	650	C57BL/Ka (25×10^6)	0/26	—	—	—	NT
C3H	650	Chimera (25×10^6 anti-BALB/c treated)‡	2/12	0/12	—	—	NT

NT, not tested.

* Fraction of animals with ≥16% donor-type (C57BL/Ka) PBL at least 40 d after cell transfer.

‡ Cells were pooled and treated in vitro with anti-BALB/c antiserum and C′.

§ Mean of individual values of definite chimeras was 44% donor-type cells.

None of 22 BALB/c mice given sublethal WBI and normal C57BL/Ka spleen cells survived >15 d. However, cotransfer of normal BALB/c spleen cells (20×10^6) with the C57BL/Ka spleen cells (20×10^6) protected the majority of irradiated BALB/c recipients from GVHD. 11 of 14 survived >60 d (Table II). The syngeneic inoculum presumably leads to rejection of the allogeneic cells because chimerism was not produced. However, treatment of the spleen cell mixture with anti-BALB/c antiserum and C′ eliminated the protective effect, because none of 12 survived >15 d. This result provided further evidence that the cytotoxicity method used in these studies eliminated host-type (BALB/c) cells, but allowed donor-type cells to mediate GVHD.

Development of Chimerism in Irradiated BALB/c Recipients Given Donor-Type (H-2^b) Chimeric or F_1 Hybrid Spleen Cells. 9 of 20 irradiated BALB/c mice (500–550 rad) given donor-type cells (25×10^6) from long-term TLI chimeras developed stable mixed chimerism (Table II). The PBL of the adoptive recipients contained a mean of 44% donor-type cells when tested at least 40 d after cell transfer. The infusion of untreated chimeric spleen cells led to stable chimerism in only 2 of 9 BALB/c recipients given 500–550 rad WBI and in none of the recipients given 450 rad (Table I). The data suggest that rejection of allogeneic cells by the lightly irradiated hosts may account for the failure to achieve uniform chimerism. The more effective transfer of chimerism with isolated chimeric donor-type cells suggests the possibility that host-type cells have a proliferative advantage in recipients of unfractionated spleen cells.

The transfer of tolerance with chimeric donor spleen cells could have been mediated by suppressor cells of donor origin, or alternatively, by donor antigens acting as tolerogens. The latter mechanism is more likely, because the majority of BALB/c mice given 500–550 rad WBI and (BALB/c × C57BL/Ka)F_1 spleen or BM cells (60×10^6) developed high levels of stable chimerism (Table III), and accepted C57BL/Ka but not C3H skin grafts indefinitely (M. Gottlieb, S. Strober, and H. S. Kaplan. Unpublished observations.). It would appear that in this strain combination, the injection of cell populations that cannot mediate a graft vs. host reaction (i.e., F_1 or donor-type chimeric spleen cells) produces stable mixed chimerism and tolerance in recipients given sublethal WBI.

The literature contains several examples of stable allogeneic BM chimeras in adult

TABLE III

Chimerism of BALB/c Mice Given Sublethal WBI and (BALB/c × C57BL/Ka)F_1 Spleen or BM Cells

Recipient treatment	Cell source	No. chimeras/ No. mice tested*	Mean percentage of chimerism‡
rad			
450	F_1 spleen (60×10^6)	0/11	—
500–550	F_1 spleen (60×10^6)	27/29	67
500–550	F_1 BM (60×10^6)	4/4	55

* Fraction of animals with ≥16% donor-type (C57BL/Ka) PBL at least 40 d after cell transfer.

‡ Mean of individual values of definite chimeras.

mice and rats prepared with lethal WBI or cyclophosphamide. These chimeras have been constructed in F_1 hybrids by infusion of parental BM cells treated with anti-Thy-1 antiserum and C′, or in completely allogeneic combinations by selection of donor-recipient strains where a substantial proportion of recipients survive GVHD (8–11). Lymphocytes from lethally irradiated F_1 hybrid recipients of treated BM from one parent proliferate in response to cells of the other parent in vitro (MLR), but do not produce specific cell-mediated lympholysis (CML) (8). Suppressor cells of the CML could not be demonstrated, suggesting that the clone of cytotoxic lymphocytes responsive to the host tissues had been deleted. However, studies in fully allogeneic rat chimeras that survive transient GVHD suggest that suppressor cells maintain the tolerant state (11). Multiple mechanisms may be operative in these instances of graft vs. host tolerance as seems to be the case for neonatal tolerance (12).

Our experiments in progress attempt to determine whether graft vs. host tolerance in TLI chimeras is mediated by antigen-specific suppressor T cells of donor origin or by deletion of specific host-reactive lymphocyte clones. An ongoing role for serum blocking factors in chimeras is less likely because washed donor-type spleen cells in the absence of chimeric serum led to sustained chimerism without GVHD in BALB/c mice given sublethal WBI.

Summary

BALB/c mice given allogeneic (C57BL/Ka) bone marrow cells after total lymphoid irradiation become stable chimeras with ~80% donor-type and 20% host-type cells in the spleen. The chimeras do not develop graft vs. host disease (GVHD). Purified cells of C57BL/Ka origin from the chimeras mediated GVHD in lightly irradiated C3H (third party), but not in BALB/c (host-strain) mice. Thus graft vs. host tolerance in the chimeras could not be explained by complete immunodeficiency of donor-type cells, serum blocking factors, or suppressor cells of host (BALB/c) origin. Clonal deletion or suppression of lymphocytes reactive with host tissues remain possible explanations. The transfer of donor-type chimeric spleen cells to BALB/c recipients given 500–550 rad whole-body irradiation WBI led to stable mixed chimerism in ~50% of recipients. The cells were presumably acting as tolerogens because similarly irradiated BALB/c mice given (BALB/c × C57BL/Ka)F_1 spleen or bone marrow cells also became stable mixed chimeras.

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References

1. Slavin, S., S. Strober, Z. Fuks, and H. S. Kaplan. 1977. Induction of specific 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.
2. Slavin, S., Z. Fuks, H. S. Kaplan, and S. Strober. 1978. Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. *J. Exp. Med.* **147:**963.
3. Slavin, S., B. Reitz, C. P. Bieber, H. S. Kaplan, and S. Strober. 1978. Transplantation tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart, and marrow allografts. *J. Exp. Med.* **147:**700.
4. Strober, S., S. Slavin, M. Gottlieb, I. Zan-Bar, D. P. King, R. T. Hoppe, Z. Fuks, F. C. Grumet, and H. S. Kaplan. 1979. Allograft tolerance after total lymphoid irradiation (TLI). *Immunol. Rev.* **46:**86.
5. Gottlieb, M., S. Strober, R. T. Hoppe, F. C. Grumet, and H. S. Kaplan. 1980. Engraftment of allogeneic bone marrow without graft-versus-host disease in mongrel dogs using total lymphoid irradiation (TLI). *Transplantation. (Baltimore).* **29:**487.
6. Slavin, S., and S. Strober. 1979. Suppressor mechanisms in tissue transplantation tolerance following total lymphoid irradiation. *In* T and B Lymphocytes: Recognition and Function F. Bach, B. Bonavida, E. S. Vitetta, and C. F. Fox, editors. **16:**241.
7. Strober, S., M. Gottlieb, S. Slavin, D. P. King, R. T. Hoppe, Z. Fuks, C. P. Bieber, and H. S. Kaplan. Immunosuppression and tolerance after total lymphoid irradiation. *Transplant. Proc.* In press.
8. Sprent, J., H. von Boehmer, and M. Nabholz. 1975. Association of immunity and tolerance to host *H-2* determinants in irradiated F_1 hybrid mice reconstituted with bone marrow cells from one parental strain. *J. Exp. Med.* **142:**321.
9. Vos, O., and W. W. H. Weyzen. 1962. A specific immunological tolerance in radiation chimeras. *Transplant. Bull.* **30:**507.
10. Gengozian, N., T. Makinodan, C. C. Congdon, and R. D. Owen. 1958. The immune status of long-term survivors of lethally x-irradiated mice protected with isologous, homologous, or heterologous bone marrow. *Proc. Natl. Acad. Sci. U. S. A.* **44:**560.
11. Tutschka, P., R. Schwerdtfeger, S. Slavin, and G. Santos. 1977. Mechanism of donor to host tolerance in rat bone marrow chimeras. *In* Experimental Hematology Today S. J. Baum and G. D. Ledney, editors. Springer-Verlag New York, Inc., New York. 191.
12. Hašek, M., and J. Chutná. 1979. Complexity of the state of immunological tolerance. *Immunol. Rev.* **46:**3.