Brief Definitive Report

REJECTION OF BONE MARROW ALLOGRAFTS BY MICE WITH SEVERE COMBINED IMMUNE DEFICIENCY (SCID)

Evidence that Natural Killer Cells Can Mediate the Specificity of Marrow Graft Rejection

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Unimmunized inbred and F_1 hybrid mice have the ability to reject allogeneic or parental bone marrow grafts after lethal irradiation (1, 2). The determinants on the hemopoietic stem cell recognized during marrow rejection have been termed Hybrid or Hemopoietic histocompatibility (Hh) antigens. The effector cell mediating marrow graft rejection bears many similarities with the cells mediating natural killer cell (NK cell) activity (3). However, in attempting to understand the generation of the specificity seen in bone marrow graft rejection, the conventional view of NK cells as nonspecific killers presents difficulties. NK cells seem to be improbable mediators of specificity, as they are thought to recognize as yet undefined target structures on tumor cells or virally infected cells in an MHC-unrestricted manner (4). It was recently proposed that NK cells do not use specific receptors, but rather interact with target cells through the use of Fc receptors and immunoglobulin. Evidence was presented indicating that the serum of mice contains "natural antibodies" to Hh or to other H-2 antigens on incompatible marrow cells, which can then bind to stem cells thus rendering them susceptible to NK cell–mediated antibody dependent cell mediated cytotoxicity (ADCC) (5). According to this view then, specificity is not intrinsic to NK cells but is conferred on them by cell bound antibodies.

To test this hypothesis, we used antibody-deficient C.B-17 *scid* mice, which are homozygous for the *scid* gene and display a severe combined immune deficiency (SCID) syndrome. They lack both T and B cell functions but display normal NK activity (6, 7). SCID mice lack detectable circulating immunoglobulins and therefore any potential "natural antibodies." These mice provide a useful model to test the hypothesis that antibodies are needed to mediate the specificity seen in marrow graft rejection by sensitizing stem cells for NK cell–mediated ADCC.

Materials and Methods

Mice. C.B-17/ICR mice homozygous for the *scid* mutation, designated C.B-17 *scid*, and C.B-17 controls, both H-2^d, were kind gifts from Dr. Melvin Bosma of the Institute for Cancer Research, Philadelphia, PA. The mice were kept in specific pathogen–free conditions until use. Mice were used at 8–16 wk. C57BL/6 (B6) (H-2^b), B6D2F1 (H-2^b/

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H-2^d), and DBA/2 (H-2^d) mice were bred in a colony at the University of Texas Health Science Center.

Irradiation. Recipient mice were exposed to 450–800 cGy of ^{137}Cs gamma radiation at a dose rate of 85.5 cGy/min. The exposure doses were: SCID mice and C.B-17 mice, 550 cGy; DBA/2 and C57BL/6 mice, 750 cGy; and B6D2F_1 mice, 800 cGy.

Treatment with Poly(I:C). Mice in some groups were injected intraperitoneally with 120 μg of poly(I:C) (P-L Biochemicals, Inc., Milwaukee, WI) 1 d before marrow cell transfer to stimulate interferon secretion in order to boost marrow allograft reactivity (3).

Assays for Marrow Cell Proliferation. The standard assay for proliferation of grafted hemopoietic cells in lethally irradiated mice has been described previously (1, 2). Briefly, in the one-step assay, prospective recipient mice were lethally irradiated and infused with inocula of 4–5 × 10^6 bone marrow cells (BMC) i.v. The proliferation of donor cells in the spleens of recipients was assessed 5 d later by measuring the incorporation of 5-[^{125}I]iodo-2′-deoxyuridine (^{125}I-UdR). Each mouse was injected with 10^{-7} M fluorodeoxyuridine i.p. to inhibit endogenous thymidylate synthetase. 1 h later each mouse was injected with 0.5 μCi ^{125}I-UdR (Amersham Corp., Arlington Heights, IL). 18 h later, the mice were bled, sacrificed, and their spleens were removed and placed in counting vials. ^{125}I radioactivity was measured in a well-type gamma counter. The data are presented as the geometric means (with range of 95% confidence limits) of percentage of injected ^{125}I-UdR incorporated into spleens of groups of 5–8 mice. Irradiated syngeneic recipients were used to assess the growth potential of the grafted BMC (syngeneic controls). The spleens of lethally irradiated mice not injected with BMC retained 0.01–0.03% of the injected isotope (radiation controls). In two-step experiments, BMC were infused as before into the lethally irradiated primary recipients. However, at days 5 or 6, the spleens of the primary hosts were removed and cell suspensions were prepared and washed in RPMI 1640 medium. Secondary irradiated hosts, syngeneic with the original marrow donors, were infused with inocula of one-fifth spleen cell equivalents. The isotope assays were performed 5–7 d after spleen cell retransplantation as described above. ^{125}I-UdR uptake in the secondary hosts reflects the generation of new progenitor cells in the spleen of the primary hosts (1, 2).

Testing C.B-17 scid Serum for Circulating Immunoglobulin. Mice were bled from the lateral tail vein before sacrifice. Serum immunoglobulin levels were kindly determined by Tracy Stevens in the laboratory of Dr. Ellen Vitetta (Dept. of Microbiology, Univ. of Texas Health Science Center at Dallas) using an isotype-specific radioimmunoassay, as described previously (6).

Production of Chimeras. C.B-17 *scid* BMC were flushed from the femurs and tibias or were released by gently crushing the backbone. The cells were suspended in RPMI 1640 medium. Cell suspensions were washed twice and placed on ice at a final concentration of 2 × 10^7 cells/ml. C57BL/6 mice were treated with 40 μl anti-asialo GM_1 serum (Wako Chemicals, Dallas, TX) i.v. to eliminate marrow allograft reactivity (8) and were irradiated (750 cGy). Within 2 h of irradiation, marrow cells were infused into the lateral tail vein in a total volume of 0.5 ml. Mice were later tested for chimerism and were used in bone marrow transplantation experiments 3–4 wk later.

Results

SCID Mice Are Capable of Specifically Rejecting Bone Marrow. A one-step splenic ^{125}I-UdR uptake assay was first performed to examine the ability of SCID mice to reject the growth of allogeneic BMC grafts. Groups of irradiated H-2^d C.B-17 *scid* mice were injected with inocula of 5 × 10^6 H-2^b B6 (Hh-1^b-incompatible), DBA/2 (H-2^d-identical but minor Hh-DBA incompatible), B6D2F1 (H-2 semiallogeneic but Hh-1-negative) or C.B-17 *scid* (syngeneic) BMC. Growth of donor-derived cells was assessed by measuring splenic ^{125}I-UdR uptake 5 d after cell transfer. SCID mice rejected B6 marrow allografts as indicated by the low splenic ^{125}I-UdR uptake (Fig. 1). Surprisingly, even H-2-identical DBA/2 marrow stem cells, which differ from C.B-17 mice with respect to minor Hh

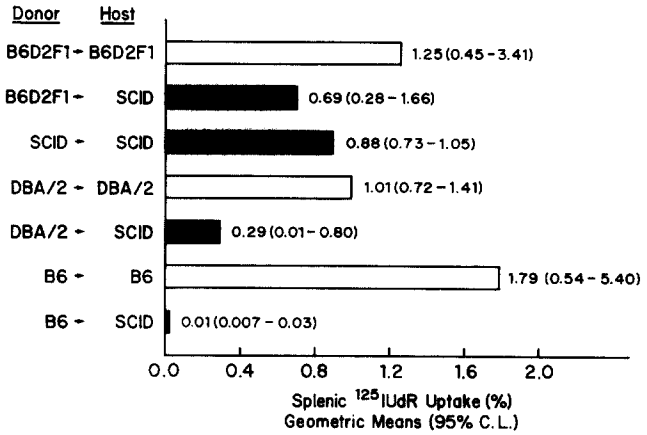

FIGURE 1. Growth of bone marrow inocula (5×10^6 cells) in C.B-17 *scid* recipients (groups of five mice, one-step assay). The DBA/2 and C57BL/6 BMC grew significantly better ($p < 0.05$) in syngeneic than in SCID hosts, whereas the growth of B6D2F$_1$ BMC in syngeneic recipients was not significantly different from that in SCID hosts.

antigens, were significantly resisted at the inocula given. Both C.B-17 *scid* and the Hh-negative B6D2F1 marrow cells proliferated to a similar extent in the SCID recipients as compared with the level of growth of the marrow in syngeneic hosts.

Two-step assays were then performed because the assay tends to be more sensitive than the one-step assay in detecting rejection, and because more parameters could be examined with the limited numbers of SCID mice that were available (Table I). In these studies, marrow growth in the primary recipients is reflected by the ^{125}I-UdR uptake in the secondary recipients, which are syngeneic with the original marrow donor. These experiments also demonstrated that allogeneic B6 marrow grafts can be resisted by SCID primary recipient mice. Furthermore, it was demonstrated that treatment of the recipient SCID mice with antisera to asialo-GM$_1$ could abrogate resistance to the marrow grafts to a significant degree. Serum immunoglobulin levels were examined after BMC transfer. Of the 15 SCID mice used as primary recipients in the two-step experiments, only three had any detectable immunoglobulins levels, which were at 0.1% of the normal C.B-17 levels. The IgG2a isotype was never detected; however, two SCID mice had 60–140 μg/ml of the IgG3 isotype (1% of normal levels) of the recipients in the two-step assays.

Adoptive Transfer of Marrow Rejection Specificity Using SCID → B6 Chimeras. To examine whether the rejection specificity to BMC grafts could be adoptively transferred, radiation bone marrow chimeras were created by infusing SCID mouse marrow cells into irradiated B6 hosts previously treated with anti-asialo-GM$_1$ serum. Such chimeras would be expected to contain NK cell activity of the SCID donor with no donor B or T cell function (9). Indeed, spleen cells of these chimeras did not respond to the B cell mitogen, LPS, (mean ± SEM [^{3}H]-thymidine incorporation (cpm), 0 ± 68 compared to 8,204 ± 3 by normal B6 spleen cells). Antibody and complement typing of the splenic effector cells in a NK cell assay against YAC-1 targets revealed that the chimeras possessed NK cells of C.B-17 *scid* (H-2^d) donor origin (data not shown).

SCID → B6 marrow chimeras were capable of rejecting host-type B6 (Hh-1^b)

TABLE I

Ability of Irradiated C.B-17 scid Mice to Reject Allogeneic Bone Marrow Cells

Exp.*	Marrow donor		Primary recipient		Secondary recipient	Splenic ^{125}I-UdR uptake	
	Strain	Number of cells grafted ($\times 10^6$)	Strain‡	n	Strain	Geometric mean	Range§
						%	
1	B6	4	B6	3	B6	0.22	0.14–0.32
	B6	4	C.B-17	3	B6	0.02‖	0.01–0.03
	B6	4	C.B-17 *scid*	3	B6	0.01‖	0.009–0.02
2	B6	4	B6	3	B6	0.19	0.09–0.24
	B6	4	C.B-17	3	B6	0.03‖	0.02–0.05
	B6	4	C.B-17 *scid*	3	B6	0.009‖	0.006–0.01
3	B6	4	B6	3	B6	1.33	0.86–2.06
	B6	4	C.B-17	3	B6	0.02‖	0.01–0.03
	B6	4	C.B-17 *scid*	3	B6	0.01‖	0.004–0.05
4	B6	5	B6	1	B6	0.99	0.21–4.66
	B6	5	C.B-17 *scid*	1	B6	0.008‖	0.002–0.02
5	B6	5	B6	2	B6	0.42	0.18–0.75
	B6	5	C.B-17 *scid*	2	B6	0.007‖	0.001–0.04
	B6	5	C.B-17 *scid*¶	3	B6	0.22**	0.06–0.78

* Primary recipient mice in Exp. 1–3 received poly(I:C) on the day of marrow cell transfer.
‡ Serum immunoglobulin levels were tested by radioimmunoassay in the 15 primary SCID recipients of Exp. 1–5. Three were positive at levels of 0.1% of control C.B-17 values.
§ 95% confidence limits.
‖ Geometric mean values significantly less ($p < 0.05$) than syngeneic controls.
¶ Primary recipient mice received asialo-GM_1 antiserum on the day of marrow transfer.
** Geometric mean values significantly higher ($p < 0.05$) than without treatment with asialo-GM_1 antiserum.

marrow grafts as detected by two-step ^{125}I-UdR uptake assays (Table II). Furthermore, this rejection ability could be abrogated by treatment of the recipients with anti–asialo-GM_1 serum. These results indicate that Hh-1^b-specific marrow allograft reactivity can be transferred by a stem or progenitor cell source of NK cells incapable of producing B or T cells.

Discussion

The data presented here demonstrate that irradiated SCID mice are quite capable of rejecting bone marrow allografts (Fig. 1, Table I). This provides direct evidence that the specific rejection of marrow can occur in the absence of detectable immunoglobulins. Furthermore this ability could be adoptively transferred by placing SCID bone marrow cells into irradiated C57BL/6 hosts (Table II). The fact that SCID → B6 chimeras rejected B6 (Hh-1^b) BMC provides a powerful argument against the involvement of antibodies in determining the specificity of marrow allograft reactivity. The B6 mice would not be expected to have antibodies directed towards self (H-2)-Hh antigens, and the transferred SCID BMC would not reconstitute the chimera with immunoglobulin-producing B cells. Thus it appears that the recognition of Hh antigens on B6 stem cells by C.B-17 *scid* mice and by SCID → B6 chimeras would have been accomplished by C.B-17 *scid* NK cells, which may possess cell surface anti-Hh-1^b receptors. Whether the putative anti-Hh receptors are present only on a subpopulation of NK cells or are present on all NK cells remains to be determined. While these experiments provide strong evidence that immunoglobulin is not required for

TABLE II

Ability of C.B-17 scid → B6 Chimeras to Reject Bone Marrow Allografts

Exp.*	Marrow donor		Primary recipient		Secondary recipient	Splenic ^{125}-UdR uptake	
	Strain	Number of cells grafted ($\times 10^6$)	Strain	n	Strain	Geometric mean	Range‡
						%	
1	B6	5	B6	1	B6	0.11	0.01–1.45
	B6	5	C.B-17 *scid* → B6	1	B6	0.02§	0.002–0.36
2	B6	5	B6	2	B6	0.41	0.32–0.65
	B6	5	C.B-17 *scid* → B6	2	B6	0.03§	0.008–0.11
3	B6	5	B6	1	B6	1.81	1.17–2.79
	B6	5	C.B-17 *scid* → B6	1	B6	0.11§	0.01–0.80
4	B6	5	B6	2	B6	0.78	0.38–1.62
	B6	5	C.B-17 *scid* → B6	2	B6	0.09§	0.04–0.20
	B6	5	C.B-17 *scid* → B6‖	2	B6	0.42¶	0.24–0.75

* Primary recipient mice in experiments 1 and 2 received poly(I:C) 18 h before marrow cell transfer.
‡ 95% confidence limits.
§ Geometric mean values significantly less ($p < 0.05$) than syngeneic controls.
‖ Primary recipient mice received asialo-GM_1 antiserum intravenously on day of transfer.
¶ Geometric mean values significantly higher ($p < 0.05$) than without treatment with asialo-GM_1 antiserum.

bone marrow rejection, they do not rule out the possibility that natural antibodies may play some role in normal mice. Certainly, the conventional view of NK cells as nonspecific effector cells needs to be modified, because these results indicate that NK cells are capable of distinguishing between self-Hh and foreign Hh (B6, DBA/2) antigens, and between Hh^+ (B6) and Hh^- (B6D2F1) stem cells. The ability of SCID mice to reject BMC also suggests that the receptors used by the NK cells are not similar to B or T cell receptors, because productive rearrangements of immunoglobulin and T cell receptor genes fail to occur in SCID mice (10).

It is interesting to note that in the SCID → B6 chimeras, the donor SCID NK cells fail to become tolerized to future marrow grafts of host type, as might have been expected from previous studies using radiation chimeras (11). The lack of tolerance induction may be due to the inability of the SCID BMC to give rise to functional T cells. Thus, SCID → B6 radiation chimeras may also provide some insight as to the regulatory mechanisms of marrow reactivity.

In light of the recent progress in reducing graft-vs.-host disease in human bone marrow transplantation, solving the problem of graft rejection may become very important in determining the future success of this treatment procedure. Peter et al. (12) recently reported that haploidentical bone marrow transfers into human SCID patients resulted in incomplete engraftment in those patients with NK cell activity. This clinical study suggests that the murine SCID model may be useful in examining the issues involved in human bone marrow transplantation.

Summary

C.B-17 *scid* ($H\text{-}2^d$) mice are homozygous for the gene that causes severe combined immune deficiency (SCID). These mice have no T or B cell function, yet display normal natural killer (NK) activity. Irradiated SCID mice were

challenged with marrow grafts to determine if antibodies are necessary for marrow allograft rejection. SCID mice rejected H-2/Hh-1 allogeneic marrow grafts. Moreover, this rejection capability could be adoptively transferred using SCID marrow as a source of NK progenitors infused into irradiated B6 ($H\text{-}2^b$) hosts. We conclude that NK cells can mediate marrow allograft reactivity in the absence of immunoglobulin. It follows that NK cells probably have specific receptors for Hh antigens.

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