

BONE MARROW GRAFT REJECTION  
AS A FUNCTION OF ANTIBODY-DIRECTED  
NATURAL KILLER CELLS

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The immunological mechanisms responsible for the recognition and rejection of allogeneic bone marrow transplants have remained obscure for many years. While it was initially assumed that all transplanted allogeneic tissues are eliminated by the same cell-mediated effector mechanisms, it soon became apparent that the rejection of bone marrow allografts may involve different cellular mechanisms than those responsible for rejection of skin allografts. The key observation was that relatively small numbers of bone marrow cells transplanted into lethally irradiated T cell-deficient nude mice (1) or normal mice (2, 3) are rejected, whereas lethally irradiated beige mice, which lack natural killer (NK)<sup>1</sup> cell activity (4–8), fail to reject marrow grafts (6, 7). This led to the hypothesis that NK cells may be responsible for the rejection of relatively small grafts of allogeneic or semiallogeneic bone marrow by irradiated recipients (9). This hypothesis obtained strong support from our recent demonstration that injection of cloned NK cells into NK-deficient mice restores the ability of these mice to reject bone marrow allografts (7, 10). One observation presents a paradox, however: NK cells exhibit no H-2-specific target cell lysis *in vitro* (11), even though bone marrow graft rejection *in vivo* is exquisitely H-2 specific. The hemopoietic histocompatibility (Hh) determinants recognized during marrow graft rejection map primarily to the H-2D (12, 13) or H-2K regions (14) of the murine major histocompatibility gene complex (MHC). Therefore, if NK cells are in fact the mediators of bone marrow graft rejection in irradiated mice, the mechanisms by which they perform this specific function must be elucidated.

Here we report that the apparent paradox between the finding that NK cells are not H-2 specific *in vitro*, but cause H-2-specific marrow graft rejection *in vivo*, can be explained on the basis of the ability of NK cells to lyse targets in an antibody-dependent cell-mediated (ADCC)-type reaction. We show that there is a target-specific natural antibody in responder mice, which, in conjunction with NK cells, causes specific bone marrow graft rejection.

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<sup>1</sup>*Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; Hh, hemopoietic histocompatibility; MHC, major histocompatibility complex; NK, natural killer cells; PBS, phosphate-buffered saline.

### Materials and Methods

*Mice.* The following mouse strains, all bred in our animal facility were used: C57BL/6J, C57BL/6 beige (bg/bg), C57BL/6 (bg/+), BALB/c, (C57BL/6 × C3H)F<sub>1</sub>, and (C57BL/6 × C3H)F<sub>1</sub> (bg/bg). C3H/He, DBA/2, (C57BL/6 × DBA/2)F<sub>1</sub>, 129/J, 129/ReJ, and 129/SvJ mouse strains and the original breeding pairs for C57BL/6 (bg/bg) and C3H (bg/bg) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The recombinant strains B10, B10.D2, B10.A(18)R, and D2.GD were purchased from the Scripps Clinic and Research Foundation Vivarium (La Jolla, CA).

*Cell Lines.* The isolation and characterization of NK cell clones have been described in detail elsewhere (11, 15, 16). For all studies, the C57BL/6 NK clones, NKB61A2 and NKB61B10 were used. Both clones were propagated in conditioned media prepared from cell supernatants of concanavalin A-stimulated normal spleen cells (Con A SN) (11, 15, 16). The following tumor cells, propagated in vitro in Dulbecco's minimal essential medium supplemented with 10% horse serum were used as target cells for cytotoxicity assays: the C3H myeloma C1.18.4 (H-2<sup>k</sup>), the BALB/c RAW 253 (H-2<sup>d</sup>) (R. Hyman, Salk Institute, San Diego, CA), and the C57BL/6 thymic lymphoma EL4 (H-2<sup>b</sup>).

*Bone Marrow Transplantation.* As bone marrow recipients, either normal mice, mice carrying the bg/bg mutation (i.e., NK-deficient) (4) or mice treated with four weekly doses of 200 rad  $\gamma$  irradiation (i.e., NK-deficient) were used (17). Shortly before bone marrow cell transfer, recipient mice were exposed to a lethal dose (800–950 rad) of <sup>60</sup>Co  $\gamma$  irradiation. Bone marrow cells, obtained from the femur of respective donor mice, were injected intravenously into sex- and age-matched lethally irradiated recipients. Proliferation of transplanted cells was assessed 5–12 d later by measuring the incorporation of <sup>125</sup>I-uridine into splenocyte DNA (2). The dose of bone marrow, 0.5–2 × 10<sup>6</sup> cells per mouse, was adjusted for each respective donor-recipient strain combination such that upon injection of <sup>125</sup>I-uridine, ~1% of the radioactivity was incorporated into the spleens of irradiated recipient mice that had been transplanted with syngeneic bone marrow. In each experiment, the value of incorporated radioactivity determined in this syngeneic growth control was arbitrarily set at 100 U and all values in the experimental groups were normalized relative to this uninhibited bone marrow growth control. Irradiated mice not injected with marrow cells were used as negative controls for <sup>125</sup>I-uridine incorporation (~2 U). Animals with ~10 U showed no visible spleen colonies, while animals with 50–100 U had >200 spleen colonies. The mean and standard error of the arithmetic mean for each group of mice (three to six animals) are given. Groups of mice injected with syngeneic cloned NK cells received 2 × 10<sup>6</sup> cells intravenously 4–7 d before bone marrow transplantation. All data presented are from experiments assayed on day 5 after bone marrow transplantation. In some experiments, donor bone marrow cells (1–2 × 10<sup>6</sup>/ml) were incubated for 30 min at 4°C with mouse serum (1:5 vol/vol) before transplantation.

*Antibodies and Mouse Sera.* Individual mice of the respective strains were bled and their serum was collected 24 h before use in marrow transplant and ADCC experiments. Supernatants from hybridomas 11-4-1 (anti-H-2K<sup>k</sup>, IgG2a) (18) and 34-5-8 (anti-H-2D<sup>d</sup>, IgG2a) (19) were used as controls for the ADCC assays.

*Depletion of Serum Antibody by Affinity Chromatography.* Immunoglobulin (Ig) was removed from C57BL/6 mouse serum using a rabbit anti-mouse Ig agarose-coupled affinity column kindly provided by Dr. I. Trowbridge, Salk Institute, San Diego, CA. 1 ml of mouse serum was loaded on to a 10-ml affinity column, and the column was incubated for 30 min at room temperature and then washed with 0.1 M phosphate-buffered saline (PBS), pH 7.0. Ig was eluted from the column with 1 M propionic acid, pH 3.0. Collected fractions were neutralized, dialyzed against PBS, filtered, and then assayed for activity in bone marrow transplants.

*Antibody-dependent Cell-mediated and Complement-mediated Cytotoxicity Assays.* ADCC activity was assessed using a standard <sup>51</sup>Cr release assay in microtiter plates. Monoclonal antibodies or mouse sera were titrated in microtiter plates, <sup>51</sup>Cr-labeled targets (10<sup>4</sup>/well) were added to various numbers of fresh C57BL/6 splenocytes or cloned C57BL/6 NK effector cells (effector/target ratios of 100:1, 50:1, and 25:1), and the mixture (200  $\mu$ l) was incubated at 37°C in 5% CO<sub>2</sub> between 4 and 10 h. After incubation, plates were

centrifuged (400 g), 100  $\mu$ l of supernatant was removed, and released radioactivity was determined in a gamma spectrometer. The percent cytotoxicity was calculated as follows:  $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$ . Spontaneous (3%/h) and maximum release values were determined by incubation of  $^{51}\text{Cr}$ -labeled targets without effector cells in the presence of medium plus antibody or serum and 1 M HCl, respectively. ADCC assays were performed in RPMI 1640 medium supplemented with antibiotics and 0.5% bovine serum albumin (Calbiochem-Behring Corp., La Jolla, CA). For assay of complement-mediated cytotoxicity, a selected rabbit serum with low toxicity to mouse cells was used at a final dilution of 1:20.

Results

**NK Cells Cause H-2-specific Bone Marrow Graft Rejection In Vivo.** The ability of lethally irradiated mice to reject allogeneic (2) or semiallogeneic (20) bone marrow grafts of certain H-2 specificities has been noted in the past in many different strain combinations. C57BL/6 (H-2<sup>b</sup>) mice, for example, reject BALB/c (H-2<sup>d</sup>) marrow grafts but fail to reject C3H (H-2<sup>k</sup>) grafts, whereas certain 129 strain (H-2<sup>bc</sup>) mice reject C3H, but not BALB/c marrow transplants (14). (C57BL/6  $\times$  C3H)<sub>F1</sub> and (C57BL/6  $\times$  BALB/c)<sub>F1</sub> hybrid mice reject C57BL/6 parental bone marrow, but not C3H and BALB/c parental grafts, respectively. In the majority of strain combinations, the specificity of this rejection is controlled by determinants encoded in or near the D region of the H-2 gene complex (12, 14), an observation that can also be reproduced in NK-deficient C57BL/6 bg/bg mice that are reconstituted with cloned NK cells (7). In Fig. 1, for example, it is shown that C57BL/6 bg/+ mice that are heterozygous for the bg allele and

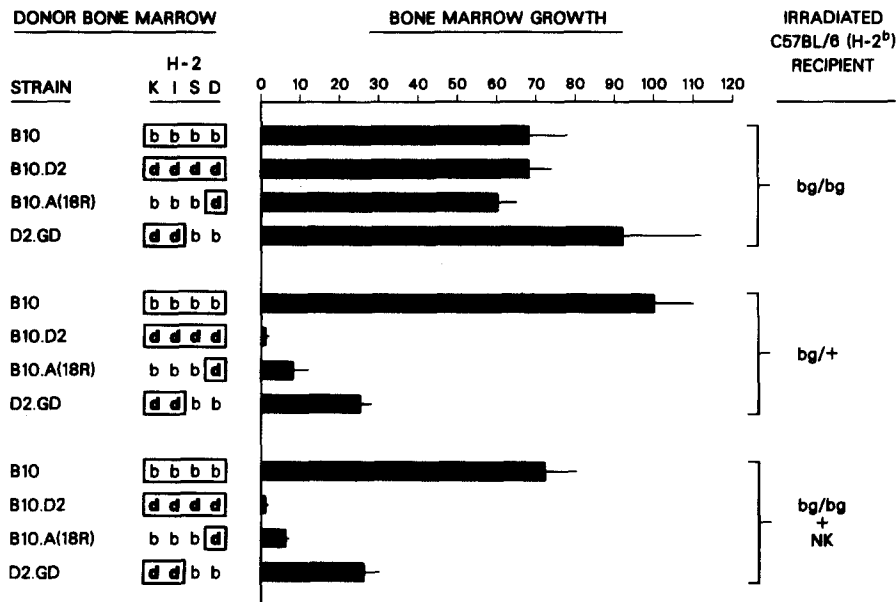


FIGURE 1. Genetic specificity of NK-mediated bone marrow allograft rejection. Lethally irradiated C57BL/6 recipient mice were transplanted with  $1 \times 10^6$  bone marrow cells obtained from H-2 recombinant donors possessing *d* alleles in various regions of the H-2 complex. NK-deficient C57BL/6 beige mice were given  $2 \times 10^6$  NKB61A2 cloned NK cells 4-7 d before marrow transplantation. Values represent marrow growth units  $\pm$  SE.

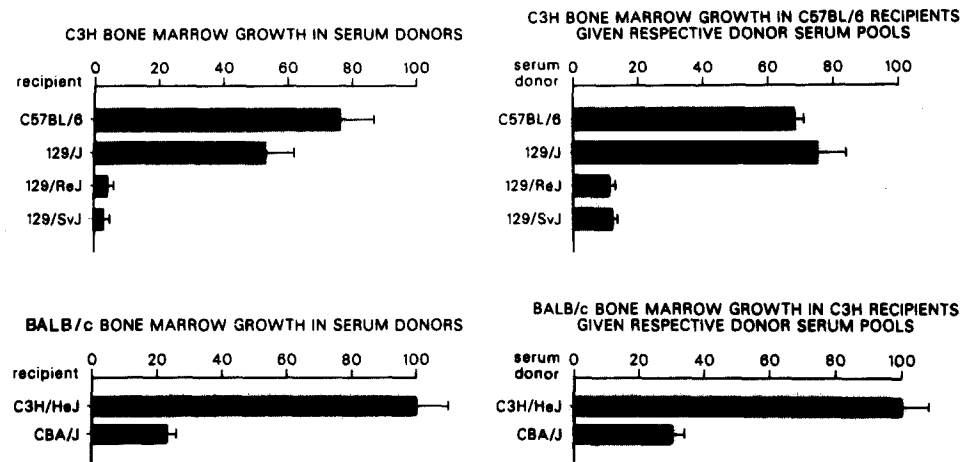


FIGURE 2. Induction of bone marrow rejection by serum transfer from responder to non-responder mice. The respective irradiated recipient mice were transplanted with either  $5 \times 10^4$  C3H or  $1 \times 10^6$  BALB/c donor bone marrow cells. In serum transfer experiments, bone marrow cells were mixed with their respective donor serum pools before injection into irradiated nonresponder recipients. Values represent marrow growth units  $\pm$  SE.

therefore possess normal NK activity reject B10.D2 ( $K^dD^d$ ) grafts and B10.A(18R) ( $K^bD^d$ ) grafts, while showing much weaker rejection of D2.GD ( $K^dD^b$ ) grafts. In contrast, NK-deficient C57BL/6 bg/bg mice do not reject marrow grafts unless they have been previously injected with cloned NK cells, in which case rejection is identical to that of bg/+ heterozygotes. This result demonstrates that cloned NK cells can induce marrow graft rejection in NK-deficient mice and that the specificity of rejection observed in these strain combinations is identical to that of normal mice.

*Ability of Mice to Reject Bone Marrow Allografts Can Be Passively Transferred by Serum from Responder to Nonresponder Mice.* Since NK cells do not show H-2 specificity in vitro (11), it was suspected that an in vivo component, such as antibody, may convey specificity. To examine this possibility, we made use of the observation that particular bone marrow allotypes grow only in one member of certain H-2-similar recipient mouse strains. For example, C3H marrow is rejected by certain 129 ( $H-2^{bc}$ ) strains but not by C57BL/6 ( $H-2^b$ ) mice (14). Similarly, BALB/c ( $H-2^d$ ) marrow is rejected by C57BL/6 and CBA ( $H-2^k$ ) but not by 129/SvJ and C3H ( $H-2^k$ ) mice (12). Therefore, experiments were designed to test whether serum collected from responder mice and injected into nonresponder mice is able to induce marrow graft rejection in nonresponders. In Fig. 2, it is seen that C3H bone marrow is not rejected by either C57BL/6 or strain 129/J mice, but is rejected by strains 129/ReJ and 129/SvJ. Sera obtained from C57BL/6 or 129/J mice and injected in conjunction with C3H marrow into nonresponder C57BL/6 recipients has little effect on donor marrow growth. However, sera from 129/ReJ and 129/SvJ cause a significant suppression of C3H marrow transplant growth in C57BL/6 recipients. In an analogous system, BALB/c marrow grafts are rejected by CBA but not by C3H mice. If the serum of either CBA or C3H is injected in conjunction with BALB/c bone marrow into nonre-

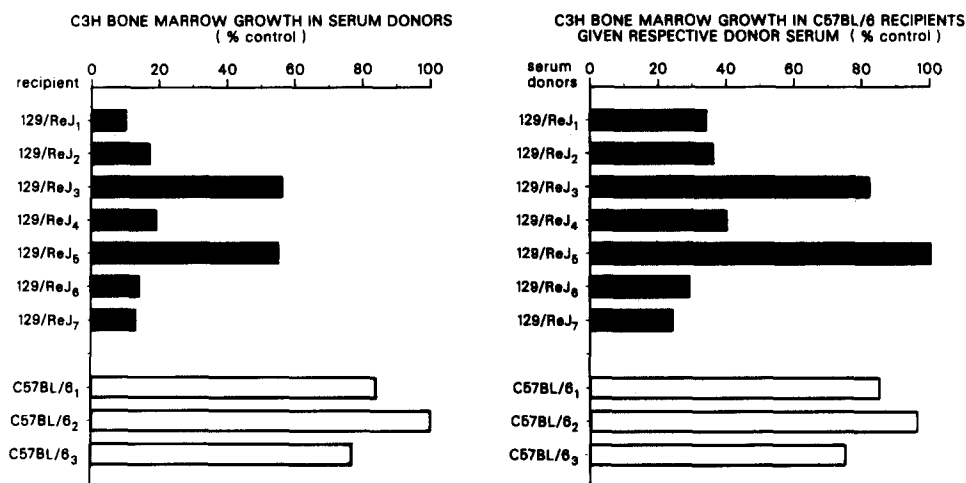


FIGURE 3. Correlation between the resistance of individual 129/ReJ mice to marrow grafts and the ability of their serum to induce graft rejection in nonresponder mice. Irradiated recipient mice were transplanted with either  $5 \times 10^5$  C3H donor bone marrow cells or bone marrow cells mixed with serum (1:5 vol/vol) from individual 129/ReJ mice.

sponder C3H mice, only the CBA serum transfer results in a suppression of BALB/c marrow transplant growth.

*Variability in Individual 129/ReJ Mice to Reject a Bone Marrow Graft Is Reflected in the Potency of Their Sera to Transfer the Ability to Reject Bone Marrow Allografts to Nonresponder Mice.* We noted during the course of these experiments that individual 129/ReJ strain mice exhibit some variation in their ability to reject C3H bone marrow transplants. It was therefore interesting to examine whether this variability correlates with the capacity of the serum from individual 129/ReJ strain mice to induce marrow graft rejection in nonresponder mice. 129/ReJ mice were bled and individually tested for C3H marrow graft rejection, and their serum was simultaneously tested for its capacity to cause C3H graft rejection in nonresponder C57BL/6 recipients. The results in Fig. 3 show that there is a striking correlation between the ability of mice to reject C3H marrow grafts and their ability to confer this capacity via their serum to nonresponder mice. It was observed, however, that serum-induced graft rejection is often not quite as efficient as the direct rejection seen in the responder serum donors (Fig. 3), which may be explained by the relatively low amount of serum (i.e., 0.1–0.3 ml) injected into recipient mice.

*Bone Marrow Rejection Induced in Nonresponder Mice by Serum Transfer Is Specific.* Since rejection of H-2<sup>d</sup> bone marrow by C57BL/6 mice is very strong (Fig. 1) and depends primarily on the expression of determinants of marrow cells encoded in the H-2D region, it was interesting to explore whether serum from C57BL/6 mice is also able to cause rejection of marrow expressing the D<sup>d</sup> allele in nonresponder recipients. Fig. 4 shows that C57BL/6 recipient mice reject B10.D2 (K<sup>d</sup>D<sup>d</sup>) and B10.A(18R) (K<sup>b</sup>D<sup>d</sup>) but not D2.GD (K<sup>d</sup>D<sup>b</sup>) grafts. In contrast, marrow transplants from B10.D2 and B10.A(18R) are not rejected by nonresponder strain 129/SvJ mice (Fig. 4). Transfer of C57BL/6 serum along with

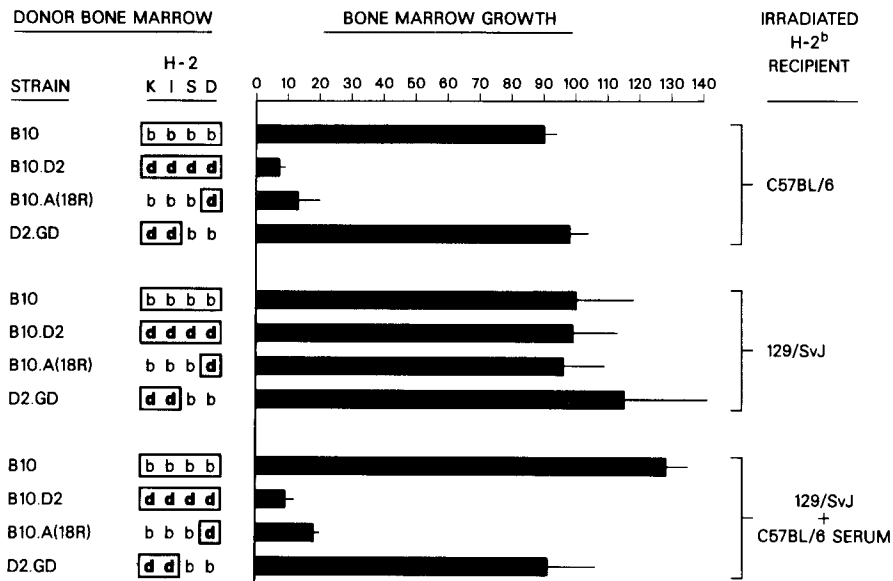


FIGURE 4. Genetic specificity of bone marrow graft rejection following serum transfer. Serum from C57BL/6 responder mice was mixed with bone marrow obtained from H-2 recombinant donor mice possessing *d* alleles in various regions of the H-2 complex. The sera (1:4 dilution) and donor bone marrow cell ( $1 \times 10^6$  cells) mixtures were injected intravenously into irradiated nonresponder 129/SvJ recipient mice and marrow growth assayed after 5 d. Donor marrow without serum was also transplanted into C57BL/6 and 129/SvJ control groups. Values represent marrow growth  $\pm$  SE.

donor marrow cells into 129/SvJ recipients results in the rejection of B10.D2 and B10.A(18R) marrow but not of D2.GD marrow (Fig. 4). These results show that marrow graft rejection is not only induced by transfer of serum from responder to nonresponder mice, but, in addition, the rejection appears to be H-2D<sup>d</sup> specific.

*Component Responsible for Induction of Marrow Graft Rejection in Nonresponder Mice by Serum Transfer Adsorbs to and Can Be Eluted from an Anti-Ig Affinity Column.* The finding that serum-induced marrow graft rejection in nonresponder mice is specific suggested that serum antibody is responsible for the graft rejection induced by serum transfer. If this were the case, then depletion of Ig from responder serum should eliminate its ability to induce marrow graft rejection in nonresponder mice. Accordingly, C57BL/6 serum was applied to a rabbit anti-mouse Ig agarose-coupled affinity column and the absorbed and nonadsorbed materials were tested for their ability to induce marrow graft rejection. The results (Table I) showed that both unfractionated C57BL/6 serum as well as eluted column-bound material (i.e., Ig) are able to induce rejection of BALB/c bone marrow in otherwise nonresponsive 129/SvJ recipient mice. In contrast, the Ig-depleted C57BL/6 serum was unable to induce marrow graft rejection. These results strongly argue that the serum component responsible for marrow graft rejection is indeed antibody.

*Responder Serum May Induce Specific Target Cell Lysis in a Complement-mediated Cytolytic Reaction.* To further substantiate the conclusion that antibody is re-

TABLE I  
*Ig-depleted Responder Serum Does Not Induce Marrow Graft Rejection*

Marrow recipient	C57BL/6 serum given to recipient*	Marrow growth†
C57BL/6	None	7 ± 2
129/SvJ	None	100 ± 14
129/SvJ	Normal	9 ± 3
129/SvJ	Ig depleted	97 ± 9
129/SvJ	Ig fraction	8 ± 1

\* BALB/c bone marrow cells were injected with either normal C57BL/6 serum, C57BL/6 serum that was Ig depleted by affinity chromatography, or the Ig fraction of C57BL/6 serum that was bound and eluted from an affinity column.

† Irradiated recipient mice were transplanted with  $1 \times 10^6$  BALB/c (H-2<sup>d</sup>) bone marrow cells and assayed 6 d later for marrow growth.

TABLE II  
*Correlation Between Graft Rejection and Serum plus Complement-mediated Lysis*

Graft recipient and serum donor	Graft and target donor	Graft rejection	Percent target cell lysis*			
			Serum dilution			
			1:4	1:8	1:16	1:32
C57BL/6	C57BL/6	—	1	1	1	1
C57BL/6	DBA/2	+	22	32	28	1
C57BL/6	C3H‡	—	1	1	1	1

\* Target lysis was assayed with rabbit complement and splenocytes that had been incubated with 10 µg/ml bacterial lipopolysaccharide for 48 h before the assay.

‡ Other experiments have shown lysis on C3H targets.

sponsible for marrow graft rejection in responder mice, sera of such mice were assayed for their ability to lyse targets in a complement-mediated reaction. We found that sera from C57BL/6 mice caused lysis of DBA/2 splenocytes, but not of C57BL/6 or C3H splenocytes in the presence of complement, a finding that correlates with the specificity of marrow graft rejection (Table II). In subsequent experiments, however, we observed that C57BL/6 sera may also occasionally lyse C3H splenocytes. This finding was unexpected, since C3H bone marrow is not rejected by C57BL/6 mice. Results discussed below provide a likely explanation in that not all antibody isotypes may be able to cause marrow graft rejection, even though they are able to effect complement-dependent cell lysis.

*Responder Serum Is Able to Induce a Specific ADCC Reaction In Vitro.* Since the ability of responder mice to reject marrow grafts can be transferred via serum antibody to nonresponder mice, one would expect that responder serum would induce a specific ADCC reaction in vitro. Individual responder 129/SvJ and nonresponder C57BL/6 strain mice were bled and tested for C3H (H-2<sup>k</sup>) marrow graft rejection, and their sera were subsequently assayed in vitro for ADCC on an H-2<sup>k</sup> tumor target. The results in Fig. 5 show that there is an absolute

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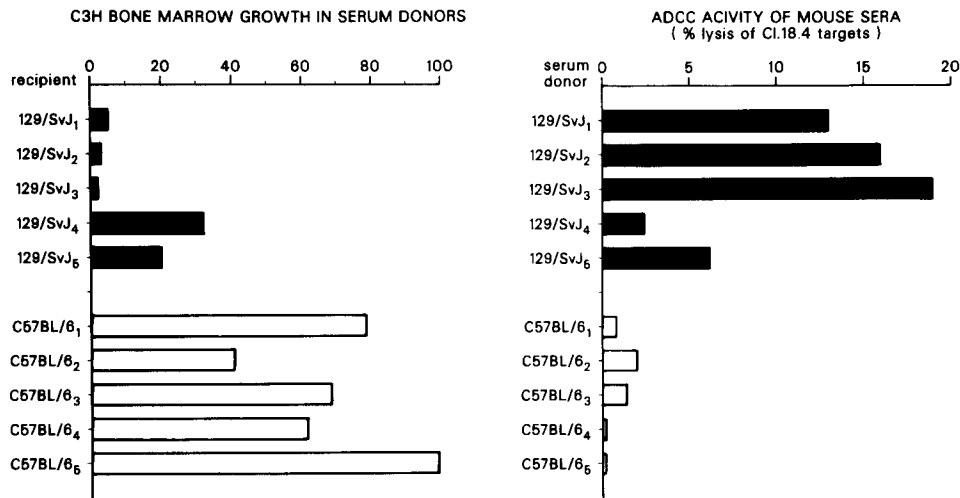


FIGURE 5. Correlation between the rejection by individual mice of H-2<sup>k</sup> marrow grafts and the ability of their serum to induce in vitro ADCC of an H-2<sup>k</sup> tumor target. Lethally irradiated recipient mice were transplanted with 5 × 10<sup>5</sup> C3H bone marrow cells 24 h after sera of individual mice were collected for ADCC assay. ADCC was performed at an effector/target cell ratio of 100:1 using C57BL/6 effector splenocytes and <sup>51</sup>Cr-labeled C1.18.4 tumor target cells in an 8-h assay; the final serum dilution was 1:8.

correlation between the ability of individual 129/SvJ mice to reject C3H marrow grafts and the capacity of their serum to induce ADCC of C1.18.4 (H-2<sup>k</sup>) targets. Furthermore, the ADCC reaction is target specific (Fig. 6) in that cytolysis of C1.18.4 (H-2<sup>k</sup>), but not RAW253 (H-2<sup>d</sup>) or EL4 (H-2<sup>b</sup>) targets, is induced by serum from 129/SvJ mice that reject H-2<sup>k</sup> but not H-2<sup>d</sup> or H-2<sup>b</sup> marrow grafts. In contrast, serum from C57BL/6 mice, which reject H-2<sup>d</sup> but not H-2<sup>k</sup> or H-2<sup>b</sup> marrow grafts, is able to induce ADCC of RAW253 targets, but not of C1.18.4 or E14 target (Fig. 6). Therefore, it is apparent that in responder mice there is a serum component, probably antibody, that induces specific ADCC with splenocytes from nonresponder mice.

*Is the Mechanism of Hybrid Resistance to Parental Marrow Grafts Similar to the Mechanism of Bone Marrow Allograft Rejection?* In the experiments described so far, induction of marrow graft rejection in nonresponder mice was demonstrated in allogeneic marrow graft recipient and donor combinations. To explore whether a similar mechanism was operating in the phenomenon of hybrid resistance, F<sub>1</sub> hybrid mice carrying the beige mutation were tested for the rejection of parental marrow grafts. The results in Table III show that (C57BL/6 × C3H)F<sub>1</sub> mice reject parental C57BL/6 but not C3H marrow grafts. However, the presence of the homozygous beige mutation in F<sub>1</sub> mice results in the growth of the C57BL/6 marrow graft. This result suggests that the rejection of parental marrow grafts involves the participation of NK cells. To explore next whether F<sub>1</sub> hybrid mice possess antibody with antiparental specificity, serum from (C57BL/6 × DBA/2)F<sub>1</sub> mice was collected because these mice show a reproducible and strong rejection of C57BL/6 but not DBA/2 grafts. Consequently, sera of these mice should be able to induce rejection of C57BL/6 grafts in nonre-



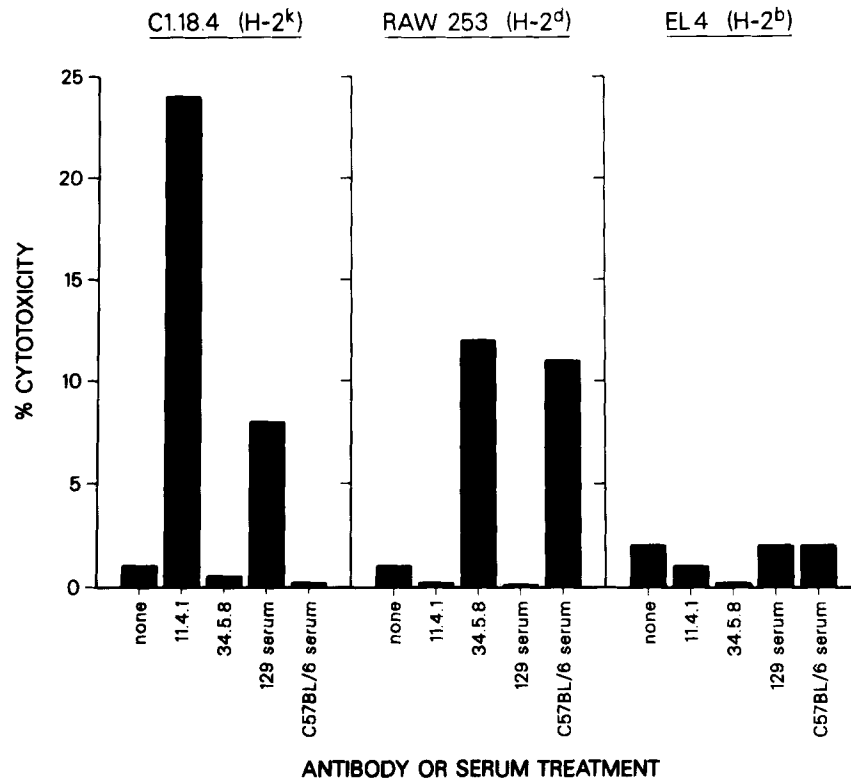


FIGURE 6. Specificity of 129/Svj strain sera in the ADCC reaction. C57BL/6 splenocytes were used as effector (effector/target ratio 100:1) and C1.18.4, RAW253, and EL4 <sup>51</sup>Cr-labeled tumor cells were targets in a 9-h cytolytic assay. Respective mouse sera, and the 11-4-1 (anti-H-2K<sup>k</sup>) and 34.5.8 (anti-H-2D<sup>d</sup>) monoclonal antibodies were used at final dilutions of 1:5, 1:10, and 1:10, respectively.

TABLE III  
Bone Marrow Graft Rejection by F<sub>1</sub> Hybrid Recipients

Bone marrow recipient*	Bone marrow donor	Serum	Marrow growth
(C57BL/6 × C3H)F <sub>1</sub>	C3H	—	86 ± 16
(C57BL/6 × C3H)F <sub>1</sub>	C57BL/6	—	3 ± 1
(C57BL/6 × C3H)F <sub>1</sub> bg/bg	C3H	—	101 ± 10
(C57BL/6 × C3H)F <sub>1</sub> bg/bg	C57BL/6	—	100 ± 4
(C57BL/6 × DBA/2)F <sub>1</sub>	DBA/2	—	85 ± 10
(C57BL/6 × DBA/2)F <sub>1</sub>	C57BL/2	—	4 ± 1
C3H	C57BL/6	—	100 ± 12
C3H	C57BL/6	(C57BL/6 × DBA/2)F <sub>1</sub> ‡	32 ± 5

\* Recipients irradiated with 800 rad γ irradiation.

‡ Injected with serum (1:2) collected 24 h before transplantation with 1 × 10<sup>6</sup> donor bone marrow cells.

sponder recipients. Since C3H mice fail to reject C57BL/6 marrow grafts, (C57BL/6 × DBA/2)F<sub>1</sub> serum was injected with C57BL/6 marrow grafts into C3H recipients. With some batches of mice, a relatively weak rejection (Table III) was observed, while in other experiments the serum had no effect (data not

shown). Therefore, in contrast to the allogeneic models, a consistent serum-mediated marrow graft rejection could not be demonstrated in the semiallogeneic system.

### Discussion

In this study, we have examined the hypothesis that specific bone marrow graft rejection involves an ADCC-type mechanism in which NK cells and specific natural antibody participate. Several approaches were used to obtain evidence for this hypothesis. We searched for a component in the serum of responder mice, which, when transferred to nonresponder mice, is able to induce specific marrow graft rejection. We found that serum from responder mice confers on allogeneic nonresponder marrow recipients the ability to reject bone marrow allografts in an H-2-specific manner. We noted, however, that graft rejection induced as a result of serum transfer was not always as efficient as that observed in direct transplants into serum donor mice. A likely explanation for this is that the natural serum antibody is limited, as we could not detect activity in responder sera diluted beyond a dilution of 1:20 in ADCC assays. Also, any limitation of natural antibody would be compounded following serum transfer, since only 0.1–0.3 ml of serum was transferred to nonresponder recipients in any given experiment. Nonetheless, our experiments clearly show that a serum component is able to induce rejection of bone marrow grafts in otherwise nonresponder recipients. We believe that antibody is responsible for this induced marrow graft rejection for several reasons. Serum from responder mice is able to induce ADCC of targets expressing the same H-2 antigens as the rejected marrow graft. By testing individual mice, we observed that there is an absolute correlation between marrow graft rejection in responder mice and the capacity of their serum to induce specific *in vitro* ADCC. Furthermore, marrow graft rejection, after serum transfer, mapped to the same H-2 region (i.e., predominantly H-2D) as that observed in normal C57BL/6 responder mice. Moreover, the capacity of responder sera to induce marrow graft rejection could be abrogated after removal of immunoglobulin by anti-Ig affinity chromatography.

There are some previous observations that would argue against the involvement of antibody in allogeneic bone marrow graft rejection. For example, it was reported that antibody could not be detected at the peak of marrow graft rejection and was not induced as a result of transplantation (2, 21, 22). Antibody induction, however, would not be expected in irradiated animals and the small amounts of preformed antibody (i.e., natural) might have been adsorbed by the transplanted tissue and thus would not be detectable at the time of transplant rejection. It may, therefore, be crucial to assay the serum of untreated responder mice for the presence of antibody, as was done in the present study. Transfer of such sera from responder mice into nonresponder mice had been attempted previously without demonstrable effects (2). A possible explanation for this failure could be that sera were injected intraperitoneally and the bone marrow transplant intravenously, while in our experiments both serum and transplant were injected together intravenously into nonresponder recipients. It is conceivable, therefore, that the previous failure to demonstrate serum-mediated marrow

graft rejection was due to insufficient amounts of antibody transferred into nonresponder mice and the inability to detect serum antibody was due to adsorption to the marrow transplant.

Our conclusion that specific serum antibody is involved in acute NK-mediated marrow graft rejection, may only hold for allogeneic marrow graft rejection models. In  $F_1$  hybrid mice, the ability to reject a parental marrow graft appears also to involve NK cells, but was not consistently reflected in the capacity of their serum to cause marrow graft rejection in allogeneic nonresponder recipients. Unfortunately, this result cannot be clearly interpreted. Rejection of parental marrow grafts in  $F_1$  hybrid mice is weaker than rejection in the allosystems, and serum transfer even in the allosystems does not always result in as strong a rejection as in the responder mice from which the serum was drawn. Also, we do not know the specificity of the putative antibody in the  $F_1$  hybrid mice, or in which other strains of mice (besides the parental, which is rejected) the respective antigen is expressed. Therefore, we do not know in which strains of mice the serum of these  $F_1$  hybrid mice should or should not be assayed. Moreover, it is not evident whether the antibody specificity is always the same or whether it may vary. We do know, however, that (C57BL/6  $\times$  C3H) $F_1$  mice immunized with an H-2<sup>b</sup> Abelson tumor are able to produce anti-H-2K<sup>b</sup>D<sup>b</sup> antibody (23), which demonstrates that  $F_1$  hybrid mice are indeed capable of producing antibodies with anti-parental specificity. There is also ample evidence that natural antibody of various specificities can be found in normal animals. For example, it is known that T cell-deficient nude mice, which exhibit high levels of NK activity also possess natural antibody of various specificities (24) and thus one could explain why nude mice specifically reject marrow transplants (1). It has also been shown that sera of normal rats, rabbits and mice possess antibody specificities that are very similar to the specificity of NK cells (25, 26).

Another interesting finding is that natural anti-tumor serum reactivity in BALB/c mice appears to correlate with their resistance to various tumors (27). Thus, antibody of the appropriate specificity appears to be present in animals that express natural resistance to bone marrow or tumor grafts in many instances. Therefore, while the evidence for the participation of antibody is persuasive in the allogeneic bone marrow transplantation systems, we have to consider the possibility of other mechanisms in the semiallogeneic  $F_1$  antiparental response of other mechanisms. Since bone marrow cells express histocompatibility antigens, it is clear that they may be stimulators and targets for specific T killer cells. This is particularly relevant in allogeneic models but may also be an important consideration in the phenomenon of hybrid resistance (28). In the present model, marrow graft rejection was assayed 5 d after recipients were lethally irradiated, which should preclude sensitization of T cells during the period of assay. On the other hand, no critical experiments have yet been done to examine whether there is indeed no sensitization of recipient T cells to the transplanted bone marrow in irradiated recipients.

Another important question is what the antibodies in responder mice recognize on the transplanted bone marrow cells and by what mechanism they induce graft rejection. Specifically, are Hh antigens indeed unique antigens that are targets

for the antibody or are they identical to common histocompatibility antigens, i.e., H-2 antigens and minor histocompatibility antigens? In a recent study (29), it was convincingly demonstrated that, besides H-2D- and H-2K-encoded determinants, H-2I-coded antigens may influence bone marrow graft rejection, which suggests that at least some of the Hh antigens are identical to H-2 antigens. Consequently, one would expect that at least in some instances the serum antibody in responder mice would be H-2. In this regard, we have demonstrated that the ability of a mouse to reject a particular marrow graft is reflected in the capacity of its serum to induce an H-2-specific ADCC reaction *in vitro*. We also used complement-mediated cytolysis of targets in an attempt to demonstrate the respective antibody specifications in responder serum. However, the results were variable, which can be explained by the hypothesis that the antibody induces an NK-dependent ADCC reaction *in vivo*.

It is well established that ADCC of nucleated targets is predominantly caused by NK cells. For instance, beige mice, as well as humans with the Chediak-Higashi syndrome, not only lack NK activity but are also deficient in ADCC activity (30, 31). The cloned NK cells recently described express Fc receptors (32; unpublished observations) and exhibit ADCC. Moreover, in single-cell assays of effector/target cell conjugates, it was observed that the same effector cell lyses both an NK-susceptible as well as an antibody-coated target (33). It therefore seems plausible to assume that the serum contained antibody in responder mice targets NK cells to be cytolytic and/or cytostatic for the transplanted bone marrow. Since IgM compared with IgG is inefficient in inducing ADCC (34; unpublished results), it is easy to see why ADCC assays detect a specificity in responder sera comparable to that in marrow graft rejection, which cannot be consistently demonstrated in the complement-mediated cytotoxic reaction.

Our demonstration that acute bone marrow allograft rejection is due to antibody-directed NK cells provides the first *in vivo* demonstration of the long-known *in vitro* ADCC reaction.

### Summary

There is conclusive evidence that acute bone marrow transplant rejection in lethally irradiated mice is caused by natural killer (NK) cells. The rejection of marrow allografts is exquisitely specific and is controlled by antigenic determinants encoded in or near the H-2 gene complex. The specificity of *in vivo* marrow graft rejection contrasts with the *in vitro* specificity pattern of NK cells in cytotoxicity assays. We therefore examined how NK cells cause H-2-specific marrow graft rejection *in vivo*. Several experimental approaches are presented that suggest that natural antibody, present in responder strains of mice, specifically directs NK cells in an antibody-dependent cytolytic and/or cytostatic reaction, resulting in marrow graft rejection. The following evidence for this mechanism is documented. The ability to reject a marrow graft can be passively transferred by serum from responder to allogeneic nonresponder mice and the specificity of rejection can be mapped within the H-2 region. Serum-induced marrow graft rejection is abrogated following depletion of immunoglobulin, and the serum of responder mice is able to induce a specific antibody-dependent cytotoxic reaction *in vitro*.

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