Delayed Activation of Quiescent Donor Hematopoietic Stem Cells in the Host Marrow Cavity by Anti-Host Monoclonal Antibody

By Michel W.J. Sadelain and Thomas G. Wegmann

Bone marrow transplantation in untreated, normal adult recipient mice usually fails. This resistance to hematopoietic stem cell engraftment is effectively overcome by non-specific conditioning agents, such as irradiation and cytotoxic drugs. These conventional conditioning agents, administered to the host before donor marrow infusion, serve several functions, most importantly, immunosuppression and myeloablation. In syngeneic or semiallogeneic recipients, donor marrow engraftment can be facilitated by the administration of anti-host major histocompatibility complex (MHC) class I antibody. In the latter procedure, the conditioning regimen consists of a single injection of anti-H-2K monoclonal antibody (MoAb) given 1 week before or along with the marrow inoculum. This form of conditioning presents two major advantages: one is to avoid the numerous undesirable radiation- and drug-related side effects, and the other is to selectively target allogeneic or semi-allogeneic host cells in the presence of donor cells. In the absence of any of the above conditioning regimens, transplantation of T cell-depleted parental marrow into adult F1 hybrid recipients does not result in significant peripheral blood chimerism (see below).

In this communication, we have studied the fate of parental donor stem cells in unconditioned F1 hosts in which the hematopoietic graft fails. This in vivo analysis was made possible by administering facilitating antibody at various time points after transplantation. The specificity of antibody conditioning, which induces chimerism by targeting host cells while sparing donor cells, permits us to detect the persistence of donor cells in the host at the time of antibody administration. We find that complete donor engraftment can still be triggered as long as a month after marrow infusion, thus establishing that donor cells in the untreated host are not eliminated, but remain functional, albeit in a resting state. This system, in which isozyme-marked parental cells are quiescent in the marrow and may be electively activated by anti-host antibody conditioning and tracked in the marrow cavity, may serve as a useful model to study hematopoietic regulation in the unirradiated animal.

Materials and Methods

Mice. Adult BALB/cCr and (BALB/cCr × C3H/HeJ)F1 hybrid (CC3F) mice were bred at the University of Alberta in the animal breeding facility. Two to three month-old mice were used in all experiments and were maintained under standard housing conditions.

Bone marrow transplantation. The antibody-facilitation protocol has been extensively described, particularly in the BALB/cCr → CC3F combination. Briefly, 15 million nucleated bone marrow cells from BALB/cCr donors were administered intravenously to unirradiated age-matched, sex-matched F1 hybrid hosts. Conditioning for transplantation consisted of a single injection of 500 μg of the anti-host MHC MoAb 16-3-1N (anti-H-2K4 mouse IgG2a). Preparation of the antibody from ascites and its quantitation have been described. Administration of an equal amount of irrelevant antibody or saline has been shown not to facilitate engraftment or affect bone marrow cellularity. Parental grafts were depleted of mature T cells by complement-mediated antibody depletion, as previously described.12 This treatment completely abrogated the in vitro proliferative response of spleen cells and bone marrow cells to Concanavalin A (data not shown). In marrow transfers to secondary hosts, unmanipulated marrow cells were harvested from exsanguinated primary F1 hosts 5 or 10 days after transplantation, washed once, and administered intravenously to secondary F1 hosts 4 hours after exposure to 9 Gy (80 Gey/mn)

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(Atomic Energy of Canada, Ottawa, ON). In certain transfers, marrow cells were coated with anti-H-2Kk MoAb by incubating 10^7 marrow cells per mL with antibody at 10 μg/mL and washed once before administration.

**Chimerism assays.** The determination of chimerism based on glucose-6-phosphate isomerase (GPI) isozyme analysis has been described. Briefly, peripheral blood samples were lysed and subjected to starch gel electrophoresis. The ratio of donor (BALB/c, GPI AA) to host (CC3F1, GPI AB) isozymes was determined by a colorimetric reaction quantified via densitometry scanning, permitting chimerism levels to be read from a standard curve. The sensitivity of this method, varying from 4% for the lower ratios of AA:AB cell mixes to 1% for higher ratios, has been described. The small amount of GPI isozyme necessary for electrophoretic separation and staining of the different bands allows the correct phenotyping of a few as 50 cells, permitting the analysis of single bone marrow colonies grown in vitro in semi-solid media. This assay has been used to show that peripheral blood chimerism is accurately reflected in the bone marrow in steady-state chimeras and to analyze engraftment kinetics within the marrow cavity. To determine the donor or host origin of colony forming unit-granulocyte/macrophage (CFU-GM) progenitor cells in the marrow at different times after transplantation, bone marrow cells were cultured in a methylcellulose medium containing 10% WEHI-3 supernatant (kindly provided by Dr D. Branch, Red Cross, Edmonton, AB), 20% fetal bovine serum (Flow Laboratories, McLean, VA), 20% IMDM (GIBCO Laboratories, Grand Island, NY) and 5 x 10^{-4} M 2-mercaptoethanol. One hundred thousand bone marrow cells per mL were cultured at 37°C in a humidified 5% CO₂-containing incubator for 6 to 8 days at 1 mL per plate in Lux-R plates (Miles Scientific, Naperville, IL). Colonies were individually picked under the microscope, lysed by freeze-thawing and individually loaded onto the gel for electrophoresis. About 50 to 60 colonies per animal were phenotyped, thus allowing an accurate measurement of the degree of donor representation within the granulocyte-macrophage progenitor colony pool at any given time point after transplantation. In radiation chimeras, chimerism was quantitated 10 weeks after transplantation. To determine and staining of the different bands allows the correct phenotypic origin between 2 and 30 days after transplantation. When antibody was given 5, 10 or 16 days after transplantation, donor representation in the CFU-GM pool was 6% 15 days after transplantation in recipient mice given antihost MoAb on day 12, 97 ± 3% when MoAb on day 10, and 98 ± 3% when MoAb on day 0. Adult CC3F1 hosts were given T cell-depleted parental marrow cells intravenously on day 0, along with or 2 weeks before intravenous administration of 500 μg of anti-H-2Kk MoAb.

*Pooled from two separate experiments.

†Chimerism was determined in each mouse by isozyme analysis of peripheral blood lysates 6 months after transplantation. Values are mean ± SD for each group.

**RESULTS**

Administration of anti-host facilitating antibody after transplantation triggers delayed donor hematopoietic engraftment. In the absence of any host conditioning, administration of T cell-depleted parental BALB/c bone marrow to adult CC3F1 recipients does not result in any detectable peripheral blood chimerism, as shown in Table 1. To examine the fate of donor hematopoietic stem cells within the host, we attempted to induce chimerism by delaying the administration of the specific anti-host conditioning after donor marrow infusion. Host cells were selectively targeted in vivo by administering anti-H-2Kk MoAb, which reacts against F1 (H-2^{da}) but not donor (H-2^{b}) cells. As shown in Table 1, a single injection of 500 μg of anti-H-2Kk antibody given along with parental marrow induces complete, long term peripheral blood chimerism. Recipient F1 mice given the facilitating antibody 2 weeks after the infusion of 15 million T cell-depleted parental marrow cells were equally well-engrafted 6 months after transplantation. This observation establishes that donor hematopoietic cells survived in the host for at least 14 days. Thus, the apparent graft failure in the unconditioned host cannot be accounted for by the elimination of all donor cells within that time period.

To understand what happens to donor cells in the untreated adult host, we examined chimerism within the marrow cavity in the days after transplantation. We used a recently described assay which accurately quantitates medullary chimerism. Individual CFU-GM progenitor cells were phenotyped for their host or donor origin at any given time, using a sensitive isozyme assay. As shown in Fig 1, donor representation in the CFU-GM pool reached 88 ± 6% 15 days after transplantation in recipient mice given facilitating antibody along with donor marrow, whereas in untreated recipients, 0 to 2% of the CFU-GM were of donor origin between 2 and 30 days after transplantation. When antibody was given 5, 10 or 16 days after transplantation, donor representation rose to about 50% of the CFU-GM pool within 4 days of antibody administration in all cases (Fig 1).

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Table 1. Administration of Facilitating Antibody 2 Weeks after Marrow Infusion Induces Complete Chimerism

<table>
<thead>
<tr>
<th>Conditioning</th>
<th>No. of Mice*</th>
<th>Chimerism†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12</td>
<td>&lt;4%</td>
</tr>
<tr>
<td>MoAb on day 0</td>
<td>10</td>
<td>98 ± 3%</td>
</tr>
<tr>
<td>MoAb on day 14</td>
<td>12</td>
<td>97 ± 3%</td>
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</table>

Adult CC3F1 hosts were given T cell-depleted parental marrow cells intravenously on day 0, along with or 2 weeks before intravenous administration of 500 μg of anti-H-2Kk MoAb.

*Pooled from two separate experiments.

†Chimerism was determined in each mouse by isozyme analysis of peripheral blood lysates 6 months after transplantation. Values are mean ± SD for each group.

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Fig 1. Medullary engraftment of donor-type CFU-GM is triggered upon delayed administration of anti-host MoAb. Adult CC3F1 mice were transplanted with 15 million T cell-depleted BALB/c bone marrow cells on day 0. Host conditioning consisted of 500 μg anti-H-2Kk MoAb given either on day 0 (C), day 5 (O), day 10 (C), day 15 (O) or day 32 (O), or no antibody at all (B). Bone marrow CFU-GM content at various time points after transplantation is plotted as the percentage of total CFU-GM that are of donor origin. Each point represents 3 to 5 mice, compiled from three different experiments. Data are expressed as mean ± SD.
The engraftment kinetics were similar for each of these time points, and, if at all different, slightly faster than in F1 hosts given donor marrow and antibody together on day 0, in which 50% chimerism was achieved after 6 days. The CFU-GM pool was virtually all of donor origin by 30 days after transplantation in all groups. Antibody administration 32 days after marrow infusion resulted in complete donor chimerism as well. Since no engraftment is detected in the absence of antibody treatment, but donor hematopoietic cells nevertheless survive for a month within the host, we next asked whether donor cells are able to seed the host marrow cavity.

Quiescent, donor hematopoietic stem cells are present in the host marrow. To examine whether donor parental hematopoietic cells seed the host marrow, bone marrow cells from primary F1 hosts were transferred to lethally irradiated secondary F1 mice. Bone marrow cells were harvested from unconditioned CC3F1 recipients 5 days after they had been given 15 million cells-depleted BALB/c bone marrow cells. Three to five million marrow cells were immediately administered to the secondary CC3F1 hosts 4 hours after exposure to 9 Gy whole-body irradiation. To enhance any engraftment by parental cells putatively present in the marrow inoculum, one group of secondary recipients received marrow cells incubated in vitro with anti-H-2Kk MoAb before infusion, thereby receiving a maximum of 3 to 5 μg of antibody; another group was given 500 μg of the antibody intraperitoneally a week after the transfer. As shown in Fig 2, chimeric hematopoietic reconstitution was observed in the presence of antibody, thus indicating that parental precursor cells were present in the primary F1 host marrow cavity. Peripheral blood chimerism was very low or absent in mice that did not receive facilitating antibody. This absence of engraftment is unlikely to be due to elimination of parental cells within 7 days of the transfer, as chimerism could be induced by administration of facilitating antibody a week after transplantation. Coating of transferred F1 cells with facilitating antibody in vitro prior to transfer induced chimerism as well. Engraftment kinetics were slower than following in vivo antibody administration, but required minimal amounts of antibody. Because chimerism was sustained 3 months after the transfer and because equal reconstitution in the erythroid and myeloid compartments was observed in the secondary hosts, it appears that primitive stem cells were transferred (Table 2). This equal reconstitution in these blood compartments favors hematopoietic reconstitution by common precursor cells rather than reconstitution by committed progenitor cells of each lineage. This interpretation is also supported by finding mixed chimerism in the thymus as well (Table 2), the lower values probably reflecting a slower turnover of the bone marrow-derived lymphoid precursor than of myeloid precursors of peripheral blood cells. A comparable reconstitution, as assessed by peripheral blood analysis in long-term secondary hosts, was achieved by transferring primary F1 host marrow harvested 10 days after parental marrow infusion. Our results therefore support the idea that quiescent primitive stem cells of donor origin were present in the host marrow cavity, but failed to proliferate or differentiate as indicated by the absence of their progeny from the marrow CFU-GM pool (Fig 1) and the peripheral blood (Table 1) in primary hosts and the virtual absence of their progeny in irradiated, otherwise untreated secondary hosts.

**DISCUSSION**

In this report, we show that parental hematopoietic cells, which apparently fail to engraft F1 hybrids in the absence of host conditioning prior to transplantation, do in fact seed the host marrow cavity. They remain quiescent but functional.

<table>
<thead>
<tr>
<th>Conditioning</th>
<th>Bone Marrow</th>
<th>WBC</th>
<th>RBC</th>
<th>Thymus</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Gy</td>
<td>&lt;4</td>
<td>&lt;4</td>
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<tr>
<td>9 Gy</td>
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<td>80</td>
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<td>70</td>
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<td>35</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>9 Gy</td>
<td>ND</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>ND</td>
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</tr>
<tr>
<td>9 Gy + MoAb</td>
<td>ND</td>
<td>83</td>
<td>79</td>
<td>ND</td>
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</table>

Secondary CC3F1 hosts were given 3 to 5 million bone marrow cells harvested from primary untreated CC3F1 mice 5 days (A) or 10 days (B) post-transplant. Before transplantation, secondary hosts were exposed either to 9 Gy irradiation alone or to irradiation plus 500 μg of anti-H-2Kk MoAb administered intraperitoneally 7 days thereafter. Chimerism in each tissue is expressed as the percentage of parental representation 10 weeks after transplantation. Each line represents chimerism levels in one mouse.

Abbreviations: WBC, white blood cells; ND, not done.
for at least a month after transplantation. This was demonstrated by selective targeting in vivo of the host bone marrow cells with an anti-MHC class I MoAb, which spares donor cells. After the delayed, specific host conditioning, engraftment was induced, resulting eventually in complete donor chimerism. By using an assay that allows us to follow the kinetics of engraftment within the marrow cavity,\textsuperscript{1,2,3} we found that donor-derived CFU-GM promptly took over the CFU-GM pool following the administration of antibody at 5, 10 or 16 days after marrow infusion. The engraftment kinetics were similar regardless of the time of antibody administration, suggesting that the donor reservoir was stable over that period. The level of engraftment was the same in at least two distinct lineages, myeloid (represented by the CFU-GM) and erythroid (represented by the peripheral red blood cells). Based on transfer experiments to secondary hosts, parental precursor cells appeared to be present within the host marrow. The presence of parental totipotent hematopoietic stem cells among the transferred cells was suggested by the comparable level of chimerism found in different blood compartments.

Assuming that the failure of parental engraftment in the lethally irradiated secondary host is not caused by the lack of "space" or growth factors, which could result in selecting against the smaller pool of parental cells present in the inoculum, the absence of any degree of chimerism in the irradiated recipients which are not given facilitating antibody is intriguing. This lack of parental hematopoietic activity is suggestive of either stem cell competition for limited special proliferative sites or a regulatory mechanism downregulating the donor stem cells. Such control is unlikely to be mediated by hybrid resistance mechanisms,\textsuperscript{14} because the delayed rescue of donor engraftment by administration of antibody 7 days after cell transfer argues against the elimination of parental hematopoietic cells by cytotoxic mechanisms. The delayed activation of parental hematopoiesis suggests that parental precursors were inhibited rather than eliminated. These observations are compatible with an active inhibition of engraftment, as we proposed earlier.\textsuperscript{10,12,13} In this hypothesis, the parental hematopoietic stem cells are capable of seeding the host marrow, but are subjected to inhibitory hematopoietic regulation. This regulatory control could persist under the conditions of the adoptive transfer if hematopoietic activity by cycling F\textsubscript{1} hematopoietic cells prevents the activation of quiescent stem cells, whether of parental or F\textsubscript{1} origin. This interpretation is supported by the finding that the adoptive transfer of antibody-coated F\textsubscript{1} cells induces complete parental engraftment (Fig 2), suggesting that the parental engraftment is indeed limited by inoculated proliferating F\textsubscript{1} hybrid cells rather than radioresistant functions present in the recipients. The lack of detectable parental engraftment in primary or secondary F\textsubscript{1} hosts in the absence of facilitating antibody suggests some cautions regarding the definition of hematopoietic graft failure. Based on this analysis within the marrow cavity, it appears that one can distinguish between the early elimination of donor cells, which would result in failure of seeding, and the failure of blood production following successful seeding, suggestive of reversible downregulation of donor hematopoietic cells. These findings suggest that, in the context of endogenous marrow repopulation, one might similarly be able to distinguish between delayed elimination of the donor cells or their downregulation. Since quiescent, isozyme-marked, donor stem cells can be selectively activated in the live animal, this model may be useful to study hematopoietic regulation within the marrow cavity in an unirradiated microenvironment. For example, the effect of various hematopoietic stimuli, including the administration of pure hematopoietic growth factors or inhibitors, may be evaluated for their effects on the recruitment of the quiescent precursors into the active hematopoietic pool. Such a model may also be useful to study the relationship between the bone marrow microenvironment and tumor cells, of hematopoietic origin or otherwise, that are present in the marrow cavity but remain in a quiescent state over a prolonged period.

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REFERENCES

10. Sadelain MWJ, Voralia M, Green DR, Wegmann TG: The role of natural suppressor and natural killer activities in resistance to
Delayed activation of quiescent donor hematopoietic stem cells in the host marrow cavity by anti-host monoclonal antibody

MW Sadelain and TG Wegmann