Effect of Graft-Versus-Host Disease on Hematopoiesis After Bone Marrow Transplantation in Mice

By Pim J. van Dijken, Jennie Wimperis, James M. Crawford, and James L.M. Ferrara

We have examined the effect of graft-versus-host disease (GVHD) on the reconstitution of donor hematopoiesis in a murine bone marrow transplant (BMT) model of GVHD to minor histocompatibility antigens. GVHD had no effect on peripheral blood counts, which normalized by 1 month after BMT, and did not affect numbers of hematopoietic progenitors in the BM, which remained decreased in all transplant recipients. Donor stem cells (colony-forming unit-spleen day 8) and stem cell self-renewal remained low in all mice for 5 months after transplant, but GVHD further damaged the stem cell compartment. Peripheral counts 1 month after transplant were supported by increased numbers of stem cells in cycle and increased splenic hematopoiesis. However, GVHD altered the pattern of extramedullary hematopoiesis, causing dramatically decreased activity in the spleen and increased activity in the liver. We conclude that GVHD further decreases hematopoietic reserve and causes damage to the donor stem cell compartment during hematopoietic reconstitution after transplant, despite unaffected progenitor frequencies and peripheral blood counts.

BONE MARROW transplantation (BMT) has become a well-established treatment modality for many malignant and other hematologic disorders. In allogeneic BMT, a major complication is graft-versus-host disease (GVHD) that occurs in up to 70% of patients causing significant mortality and morbidity. Three well-established target organs of GVHD are skin, gut, and liver. The adverse effect of GVHD on immunologic reconstitution is well documented and observed for both the T-cell and the B-cell compartments in humans as well as in mice.

GVHD can also affect hematopoiesis. Studies of experimental GVHD models in nonirradiated mice show a reduction of host hematopoietic stem cells and erythropoietic progenitors. The effect of GVHD on hematopoietic reconstitution after clinical allogeneic BMT is not dramatic, although some patients with severe GVHD have persistent thrombocytopenia that may reflect poor stem cell function. This attenuated effect of GVHD on hematopoiesis after transplant may result from the sparing of donor BM progenitors, because after transplant both the donor T cells and the donor BM cells are genetically identical to each other.

To investigate the effects of GVHD on hematopoietic reconstitution after allogeneic BMT, we have used a well-defined BMT murine model of GVHD in which recipient mice are irradiated to supralethal doses (1,100 cGy). We have correlated the clinical course of GVHD with peripheral blood (PB) counts, hematopoietic progenitor frequencies, and stem cell content. We have evaluated hematopoietic potential in detail because PB counts can be insensitive indices of hematopoiesis, and several studies have documented decreased marrow cellularity and progenitor frequency after transplant despite the full recovery of PB counts. We have also investigated the activity of the stem cell compartment by measuring both stem cell renewal and by calculating the percentage of stem cells in cycle, as well as examining the effects of GVHD on extramedullary hematopoiesis. Our results show that normal PB counts 1 month after transplant are supported by increased percentages of stem cells in cycle and by expansion of splenic hematopoiesis. GVHD prevents this splenic expansion and adversely affects hematopoietic reconstitution after transplant by further reducing the number of stem cells and their self-renewal capacity.

MATERIALS AND METHODS

Marrow harvesting and treatment with monoclonal antibody (MoAb) and complement. Female B10.BR (H-2b) mice (Jackson Laboratories, Bar Harbor, ME) were killed by cervical dislocation. Femurs and tibias were removed surgically and flushed with RPMI to which had been added 5% vol/vol fetal calf serum (FCS). A suspension of 50 x 10^6 harvested BM cells was incubated for 40 minutes at 4°C with 1 mL of anti-Thy 1.2 MoAb (undiluted supernatant of CDF23) and 1 mL of 1:10 dilution of prescreened low toxic rabbit complement (Cedarlane, Hornby, Canada) in L-15 medium. After 40 minutes at 37°C, the cells were washed and a second treatment with antibody and complement was performed in an identical manner. Finally, the anti-Thy 1.2 and complement-treated marrow was washed twice in L-15. The effectiveness of T-cell removal was determined by a limiting dilution assay, and frequencies of precursor cytotoxic T cells in treated BM were always less than 1 in 10,000. Splenocyte suspensions from these same donor animals were passed over nylon wool columns to obtain a lymphocyte population enriched for T cells (>90% Thy 1 cells) by fluorescence-activated cell sorter analysis.

Irradiation and reconstitution. Female CBA/J (H-2b) mice were lethally irradiated with a 131I source (gamma cell 40; Atomic Energy of Canada, Ottawa, Canada) to 1,100 cGy (108 cGy/minute) in two doses (600 to 500) separated by 3 to 4 hours to reduce gastrointestinal toxicity. In three separate experiments, irradiated CBA/J mice (n = 54) received either 5 x 10^6 B10.BR T-cell-depleted BM (n = 24) or 5 x 10^6 B10.BR T- BM to which 10^6 nylon wool passaged Thy 1+ splenic B10.BR cells had been added.
added (n = 30). Transplanted animals received tetracycline hydrochloride (8% solution) in their drinking water for the first 4 weeks posttransplant. Age-matched, nonirradiated B10.BR donor control animals were maintained identically and were followed for survival and weight gain. Mice were killed at 1 (n = 6), 2 (n = 13), 3 (n = 12), and 5 (n = 13) months after BMT by cervical dislocation and analyzed. Age-matched donors (n = 3 to 4) served as normal controls at each timepoint. Composite data from all normal donors (n = 14) served to generate the range of normal values represented by the open bars in Figs 1 and 2. Using the H-1 Technicon (Tarrytown, NY), automated leukocyte counts were reproducible within 10% and platelet counts within 20%. To relate multiple hematopoietic parameters to each other, during the first two experiments, the same mice were used to generate data for PB counts, BM and splenic cellularity, colony-forming unit granulocyte-macrophage (CFU-GM), spleen CFU (CFU-s), marrow self-renewal capacity of hematopoietic stem cells was measured by the methyl cellulose technique described by Iscove and Sieber.27 Briefly, cells were cultured (2.5 and 5 x 10⁶ cells from normal animals) in 35-mm Lux plastic tissue culture dishes (Miles Laboratories, Inc, Naperville, IL) in 1 mL of media containing 14% methylocellulose, 20% FCS, 1.3% bovine serum albumin (BSA), 100 μM penicillin, 100 μM streptomycin, 2 mmol/L glutamine, and 1 x 10⁻⁴ mol/L 2-mercaptoethanol; 10% (vol/vol) pokeweed-mitogen stimulated spleen cell supernatant was added to the cultures as a source of stimulating factor as described by Iscove.28 Dishes were incubated at 37°C in 5% CO₂ in air in a humidified atmosphere for 7 to 8 days. Colonies were then scored using an Olympus (Waltham, MA) inverted microscope to identify aggregates of greater than 50 cells as colonies.

Liver pathology. At 1 month after BMT, livers from each animal were fixed in phosphate-buffered formalin and sliced at 1 mm intervals, yielding six to seven slices per lobe, as previously described.27 These slices were processed for light microscopy by standard methods, and adjacent 6-μm paraffin sections stained with hematoxylin and eosin, and Masson trichrome. Slides were
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Table 1. Characteristics of Transplant Groups

<table>
<thead>
<tr>
<th></th>
<th>Donor BM</th>
<th>Survival* (%)</th>
<th>Weight (g)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 mo</td>
<td>2 mo</td>
</tr>
<tr>
<td>No GVHD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 × 10⁵</td>
<td>136 ± 31</td>
<td>100</td>
</tr>
<tr>
<td>GVHD</td>
<td>5 × 10⁵</td>
<td>138 ± 35</td>
<td>94</td>
</tr>
</tbody>
</table>

* CBA mice were irradiated and transplanted with B10.BR donor BM as described in Materials and Methods.
† T-cell-depleted BM cells.
‡ Nylon wool passaged splenocytes (>90% Thy 1⁺).
§ Calculated from the number of day-8 CFU-S produced by 5 × 10⁵ BM cells in B10.BR spleens (n = 5 to 7). Values are mean ± SE.

RESULTS

BMT and induction of GVHD. CBA/J mice were administered 1,100 cGy TBI and were transplanted with 5 × 10⁵ T-cell–depleted BM cells from B10.BR donors in three separate experiments. Mice receiving T-cell–depleted BM (n = 24) had fewer than 10 cytotoxic T-lymphocyte precursors in the total BM inoculum as measured by limiting dilution analysis (LDA) (data not shown). GVHD was induced in a second group of mice using the system of Shulman et al. Extramedullary hematopoiesis (EMH) was graded and the tissue histology was scored using the system of Shulman et al. Mean values were determined for each animal analyzed, based on two independent scoring sessions. Statistical comparisons between treatment groups were performed using one-way analysis of variance followed by Newman-Keuls multiple range tests for pairwise comparisons, after testing for normality.

Hematopoietic progenitors (CFU-GM). During the entire period of observation, the number of mononuclear cells in the BM did not differ between mice with and without GVHD. BM cellularity gradually increased to the low normal range at 5 months after BMT (Fig 2A). The frequency of CFU-GM in the marrow also did not differ between groups throughout the 5-month observation period; the progenitor frequency remained depressed (30% of normal) in all recipients at 5 months (Fig 2B). Combining these observations, the absolute number of CFU-GM in the BM ranged from 15% of normal at 1 month to 30% of normal at 5 months after BMT.

Hematopoietic stem cells and self-renewal (CFU-s and R). We also analyzed the effect of GVHD on hematopoietic stem cells. Recent data have shown that neither day-8 nor day-12 CFU-s represent pluripotent hematopoietic stem cells. We therefore chose to analyze the stem cell compartment by measuring the quantity of day-8 CFU-s and the quality of these CFU-s as defined by their R. We measured day-8 CFU-s in transplanted BM both at 1 month and at 5 months after BMT (Fig 3). CFU-s frequency in animals without GVHD was approximately 50% of normal controls, but it was only 25% of normal in the GVHD group (Fig 3A). Mice with GVHD, therefore, had half as many CFU-s as transplanted mice without GVHD (P = .01). Induction analyzed on an automated cell counter (Technicon H-1) (Fig 1). Platelet counts and absolute neutrophil counts were normal at 1 month after transplant (Fig 1B and D) and remained stable in all BMT recipients regardless of GVHD status. The total white blood counts showed a slower recovery than other hematologic parameters (50% of normal at 1 month), and the mice remained mildly anemic (Fig 1C). All transplanted mice remained mildly anemic throughout the 5-month observation period, but there were no persistent significant differences related to GVHD status.

Recovery of PB values. At several times (1, 2, 3, and 5 months) after BMT, animals were killed and blood samples were obtained by intracardiac puncture. Samples were coded and examined in a random, blinded fashion by Dr Crawford and the tissue histology was scored using the system of Shulman et al. Extramedullary hematopoiesis (EMH) was graded on a 0 through 4 scale with 0 representing none; 1, rare scattered foci; 2, occasional foci (1 to 5 per 10 high powered fields); 3, frequent foci (5 to 20 per 10 high powered fields); and 4, numerous confluent zones of EMH. Mean values were determined for each animal analyzed, based on two independent scoring sessions. Statistical comparisons between treatment groups were performed using one-way analysis of variance followed by Newman-Keuls multiple range tests for pairwise comparisons, after testing for normality.
of more severe GVHD with $10^6$ T cells in the inoculum produced even greater damage to the stem cell compartment at 1 month after transplant, producing only 8% of the day-8 CFU-s generated by normal BM (data not shown). Because day-8 CFU-s are considered to be relatively mature stem cells with limited potential for self-renewal, we also measured directly the self-renewal capacity of these stem cells by the $R_s$ assay. $R_s$ is a measure of self-renewal capacity that has been shown to correlate with the serial transplantability of BM.21,24 At 5 months after transplant, $R_s$ was four times lower in mice with GVHD than in mice without GVHD (Fig 3B), despite the fact that BM cellularity had normalized and PB counts had been stable for at least 2 months (Figs 1 and 2). As expected, $R_s$ was lower in all transplanted mice compared with normal donors. GVHD therefore stresses hematopoiesis by further reducing the numbers of donor stem cells and their self-renewal capacity even though these differences were not apparent by standard functional assays of hematopoiesis (ie, PB cell counts and the frequency of BM progenitors).

**EMH.** We reasoned that EMH might help maintain near normal values of PB cells in the face of reduced numbers of BM stem cells and progenitors after BMT. We therefore analyzed spleens of and livers of transplanted mice 1 month after BMT for hematopoietic stem cells and progenitors. Spleens from mice without GVHD contained dramatically increased numbers of CFU-s and CFU-GM (Table 2). This extramedullary expansion included twice the normal number of CFU-s and five times the number of CFU-GM colonies. Mice receiving syngeneic BMT showed similarly expanded splenic hematopoiesis as the no GVHD group (data not shown), and therefore this increased splenic activity is not uncommon early after transplant. In contrast, mice with GVHD had less than normal numbers of CFU-s and low normal numbers of CFU-GM levels in their spleens. GVHD thus prevented the normal expansion of splenic BM early after BMT.

Livers from transplanted mice and donor controls were also analyzed at 1 month after transplantation and scored for changes of GVHD by the system of Shulman et al.25 The livers from mice with GVHD had significantly higher scores than livers from normal donors or from transplanted mice without GVHD as a result of increased pathology in all the major features of this grading system.27 Surprisingly, the livers from mice with GVHD also contained significantly greater amounts of EMH than both control groups (Fig 4). GVHD thus alters the pattern of EMH after transplant, with large decreases in the spleen but an increase in the liver. Because the spleen normally expands its hematopoietic progenitors after transplant, this altered pattern is most probably pathologic and may reflect increased hematopoietic stress during GVHD.

**Cycling studies.** We reasoned that greater numbers of stem cells in cycle might also support PB counts at 1 month after transplant. A final set of experiments was therefore performed to examine the cycling activity of hematopoietic stem cells by thymidine-suicide analysis (Table 3). Transplanted animals without GVHD showed nearly twice as many stem cells in cycle compared with normals, both in the spleen and in the BM. This percentage increased even further in animals with GVHD, although this difference

### Table 2. MH In the Spleen 1 Month After Transplant

<table>
<thead>
<tr>
<th></th>
<th>Cellularity ($\times 10^6$)</th>
<th>CFU-s (per $10^6$)</th>
<th>CFU-GM (per $10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal donor</td>
<td>155 ± 14</td>
<td>20.3 ± 4.6</td>
<td>123 ± 13</td>
</tr>
<tr>
<td>No GVHD</td>
<td>142 ± 16</td>
<td>43.0 ± 8.2</td>
<td>663 ± 28</td>
</tr>
<tr>
<td>GVHD</td>
<td>111 ± 21</td>
<td>14.8 ± 2.5</td>
<td>120 ± 30</td>
</tr>
</tbody>
</table>

Spleens were harvested from normal donors ($n = 3$) and from transplanted mice (no GVHD, $n = 4$; GVHD, $n = 3$) 1 month after BMT. CFU-s (day 8) and CFU-GM were determined as described in Materials and Methods. Values are mean ± SE.
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Table 3. Percentage of Cycling Day-8 CFU-s 1 Month After Transplant

<table>
<thead>
<tr>
<th></th>
<th>BM</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal donor</td>
<td>20, 30</td>
<td>6, 21</td>
</tr>
<tr>
<td>No GVHD</td>
<td>48, 50</td>
<td>ND, 40</td>
</tr>
<tr>
<td>GVHD</td>
<td>64, 75</td>
<td>ND, 51</td>
</tr>
</tbody>
</table>

BM and spleen cells were obtained from normal donors (n = 3) and from mice with GVHD (n = 3) and without GVHD (n = 3 to 4) 1 month after BMT in each of two experiments. Cells from each group were pooled and incubated with and without ³H-thymidine and then tested for CFU-s (day 8) (n = 5 to 6 for each group) as described in Materials and Methods. Percentage of CFU-s in cycle was calculated by the formula \((x - y)/x\), where \(x\) is the number of CFU-s in cells incubated without ³H-thymidine, and \(y\) is the number of CFU-s in cells incubated with ³H-thymidine.

Abbreviation: ND, not done.

was small and not statistically significant. These increases in cycling stem cells correlate well with their reduced absolute number (Fig 3A) and are indeed likely to be a compensatory mechanism to help to maintain normal values of PB cell counts (Fig 1). Increased percentages of cycling stem cells are also consistent with reduced \(R_s\) because the self-renewal capacity is lowest in the most actively cycling stem cells. Thus, despite maintenance of normal counts during GVHD, there is continued diminution of hematopoietic reserve, ie, fewer quiescent stem cells.

DISCUSSION

GVHD is a multisystemic disease that profoundly affects both lymphoid and nonlymphoid hematopoiesis after BMT. The reconstitution of nonlymphoid hematopoiesis after allogeneic BMT has not been as extensively studied as the reconstitution of the immune system, probably because it is not usually associated with obvious clinical problems. By contrast, impaired immunologic reconstitution often results in considerable clinical sequela such as chronic immunodeficiency and susceptibility to infections.

We have used a murine BMT model of GVHD to minor histocompatibility antigens to study the effect of GVHD on nonlymphoid hematopoiesis. The three main conclusions of this study are: (1) the donor stem cell compartment remains diminished in all mice for at least 5 months after transplant, and GVHD causes further stem cell damage; (2) PB counts normalize by the first month after transplant, but the number of BM stem cells and progenitors lags behind considerably, irrespective of GVHD status; and (3) the PB counts at 1 month after transplant are normally supported by increased numbers of cycling stem cells and expanded splenic hematopoiesis. GVHD prevents this splenic expansion, thus further diminishing hematopoietic reserve, and causes extramedullary hematopoiesis in the liver.

Surprising results emerged from the analysis of the effects of GVHD on hematopoietic stem cell number and self-renewal capacity. All transplanted mice showed a decrease in the number of the BM CFU-s, and an even further reduction was associated with GVHD (Fig 3A). Day-8 CFU-s are comprised predominantly of erythroid progenitor cells, and low numbers of these CFU-s may help to explain the mild anemia of all transplanted mice during the first several months after transplant (Fig 1C). The reduction in the number of stem cells was associated with a decrease in their self-renewal capacity, measured by \(R_s\); GVHD caused further reduction (Fig 3B). Altogether, these data extend previous observations of a decrease in pluripotent hematopoietic progenitors associated with GVHD.

Several mechanisms could account for the deleterious effect of GVHD on BM stem cells after transplant. A direct immunologic effect of donor T cells on donor stem cells is highly unlikely, because they are genetically identical to each other. It should be noted, however, that the phenomenon of syngeneic GVHD is now well established in experimental models. Indirect immunologic effects are also possible. Immunologically mediated damage to the hematopoietic microenvironment, which remains of host origin after BMT, is an attractive explanation. The uneven distribution of sites of hematopoiesis after transplant and the change of this distribution under the influence of GVHD support the hypothesis that GVHD affects hematopoiesis by disturbing the microenvironment in BM and spleen. Other contributing factors, such as an altered release of cytokines that regulate hematopoiesis or stem cell damage resulting from the cachexia induced by GVHD, cannot be ruled out and deserve further study.

The recovery of normal peripheral counts despite reduced numbers of BM progenitors was initially quite surprising, particularly at 1 month after transplant. The large expansion of splenic hematopoiesis might account for the maintenance of counts after syngeneic transplantation in recipients of T-cell–depleted BM, but there was no such increase in mice with GVHD (Table 2). It is possible that mice with GVHD maintained their peripheral counts by the increased extramedullary hematopoiesis in the liver (Fig 4) as well as by the very large percentage of stem cells in cycle (Table 3). The large percentage of stem cells in active cycle means that the percentage of quiescent stem cells is reduced. This reduction of quiescent stem cells, whose self-renewal capacity is greatest, may help to account for the observed reduction in self-renewal (\(R_s\)) in the BM during GVHD.

We interpret these results to suggest a dynamic and complex interaction between GVHD and hematopoietic reconstitution after transplant. In the absence of GVHD, small numbers of stem cells proliferate and are able to sustain peripheral counts through extensive splenic EMH. The continued need to sustain peripheral counts from lower than normal numbers of stem cells keeps them actively cycling, and consequently their self-renewal capacity decreases. The advent of GVHD prevents the splenic expansion of hematopoiesis, perhaps through damage to the splenic microenvironment; in this setting, peripheral counts must be supported from fewer stem cells, forcing yet more of them into cycle, with a concomitant greater loss in self-renewal capacity. Hematopoietic reserve is further stressed and EMH now occurs in the liver. It is also possible that allogeneic T cells in the liver release hematopoietic cytokines (which has been observed in other models of

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GVHD\(^7\) and these cytokines induce stem cells to differentiate inappropriately in the hepatic parenchyma.

These observations suggest large stresses on the newly engrafted marrow, with potential exhaustion of stem cells during prolonged GVHD. In this context, it is somewhat surprising that mice with GVHD did not show decreasing platelet counts, which are often a sensitive index of stem cell function. Patients with GVHD and severe thrombocytopenia have a particularly poor prognosis,\(^1\)\(^8\) but low platelet counts in these patients are probably due to multiple factors, including infection, and not simply chronically stressed hematopoiesis. It is also possible that fewer stem cells during GVHD may lead to a lower production of pre-B and pre-T cells with a consequent negative impact on the reconstitution of the immune system.\(^1\)\(^9\) Further investigations should help to elucidate the precise nature of stem cell reduction induced by GVHD and suggest possible therapeutic interventions such as the use of recombinant cytokines.

ACKNOWLEDGMENT

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