Hematopoietic reconstitution following syngeneic bone marrow transplantation with graded doses of untreated and drug-treated bone marrow was studied in B6D2F1 mice. Granulocyte-macrophage colony-forming units (CFU-GM) and spleen colony-forming units (CFU-S) showed similar in vitro drug sensitivities. Both the speed of hematologic recovery and survival of mice transplanted with untreated or drug-treated bone marrow were directly related to the number of CFU-GM or CFU-S transplanted. Similar hematologic recovery was seen for untreated marrow transplants and treated transplants that had similar CFU-GM or CFU-S content. There is a minimum number of transplanted CFU-GM or CFU-S that allows survival of lethally irradiated mice. This number is present in a marrow transplant containing the equivalent of $5 \times 10^5$ untreated cells or producing one to two spleen colonies. There also exists a maximum value for the number of hematopoietic progenitors in a marrow graft, above which the rate of hematologic recovery following transplantation is rapid and no detectable increase in the rate is seen with increasing CFU-GM or CFU-S content. The presence of this maximum value for transplanted progenitors and variations in culture techniques are probably the reasons previous studies have not always shown a correlation between CFU-GM content and hematologic recovery after bone marrow transplantation.

**Materials and Methods**

**Animals.** Female B6D2F1 mice (Cumberland View Farms, Clinton, TN), 6 to 14 weeks of age, were used for all studies except those performed to confirm donor engraftment. In these studies male B6D2F1 mice were used as bone marrow donors and female B6D2F1 mice were the recipients. The animals were housed in sterile microisolator cages. They were fed acidified water and sterilized laboratory chow ad libitum.

**Drugs and drug incubations.** The four drugs used, 4HC, vincristine, 5-fluorouracil (5FU), and bleomycin, were studied because they have a wide range of antitumor activity and because they clinically spare the hematopoietic system. For these reasons these drugs hold promise as potential “purging” agents for autologous BMT.

**Progenitor Cell Assays Predict Hematopoietic Reconstitution After Syngeneic Transplantation in Mice**

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received 11.0 Gy of total body irradiation (TBI) from a dual 137Cs source at a dose rate of 1.18 Gy/min. The mice were then inoculated with the bone marrow via the tail vein. CFU-S were assayed by the procedure of Till and McCulloch. Briefly, mice were sacrificed at day 7 or 12, the spleens were removed, and all macroscopic colonies (small, medium, and large) were counted after fixing the spleens in Carnoy’s solution (60% ethanol, 30% chloroform, and 10% acetic acid). Colonies from random spleens were counted by three different observers, and results were similar. Both day 7 and day 12 CFU-S were performed on eight mice receiving enriched McCoy’s 5A medium without any bone marrow cells. No spleen colonies were seen. Mouse survival after 11.0 Gy of lethal TBI and BMT was defined as survival for greater than 60 days. In each reconstitution experiment four irradiated controls received no bone marrow and served as radiation-only controls. All radiation control mice died, usually between days 10 and 14. For sequential neutrophil counts in these mice, the retro-orbital sinus was punctured with a heparinized microhematocrit tube. An attempt was made to limit the blood loss to less than 0.2 mL. WBC counts were determined using an automated cell counter (Coulter Electronics, Hialeah, FL) or by hemocytometer if the count was less than 10^6 cells/mm^3. Differential counts were performed on Wright’s stained blood smears, and the absolute neutrophil count was calculated from the total WBC count and the percentage of neutrophils.

All data points were the mean of two to four separate experiments performed in quadruplicate. The number of CFU obtained from assaying 10^5 untreated bone marrow cells was established for each individual experiment and was taken as 100% recovery unless otherwise specified. The results of all transplant studies for survival were the combination of two to five separate experiments. Each individual experiment utilized four to five mice per point. Statistical comparisons were done by simple linear regression analyses with calculation of Pearson’s correlation coefficients.

Y chromosome determination by Southern blot analysis of DNA samples. Standard procedures were used in the preparation and quantitation of DNA samples of bone marrow cells. Aliquots of DNA were digested overnight with BamHI in conditions suggested by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). DNA was electrophoresed through 1% agarose gel and transferred to nylon membrane (Gene Screen, New England Nuclear, Boston). Prehybridization and hybridization were performed at 60°C. The membranes were probed with a Y chromosome-specific fragment that was kindly provided by Edward Palmer. Plasmid pY2 was digested with EcoR I and Sal I. The insert was isolated by agarose gel electrophoresis and purified by electrodialysis. Radilabeling with [32P]dCTP (Amersham 3,000 Ci/mmol) was performed by random priming with Klenow DNA polymerase to a specific activity of 0.5 to 1.0 x 10^6 cpm/µg DNA.23 Washing of filters at high stringency (0.1 x SSC, 0.1% sodium dodecyl sulfate [SDS] at 65°C) resulted in no detectable signal in female DNA samples.

To determine the relative contributions of host- and donor cell-derived material in each DNA sample, the experimental DNA (5 µg total/lane) was blotted in parallel with DNA (5 µg total/lane) from known mixtures of male and female bone marrow cells as described by Lemischka et al.21 Filters were probed with BamH I fragment of mouse c-myc, an autosomal probe, to correct for DNA loading differences and densitometric scanning of lanes was performed.

### RESULTS

Syngeneic bone marrow grafts containing between 10^5 and 10^6 untreated nucleated cells were assayed for CFU-GM, CFU-S, and the ability to rescue lethally irradiated mice. Table 1 shows that a linear relationship exists between the number of cells assayed and the number of CFU-GM and CFU-S obtained over the entire range of untreated marrow cells assayed. The results of day 7 and day 12 CFU-S from untreated marrows were the same. Table 1 also shows that the transplantation of only 5 x 10^4 untreated syngeneic marrow cells was able to rescue 1/6 lethally irradiated mice. The number of CFU-GM observed with assaying 5 x 10^4 untreated marrow cells was 5.4% ± 0.6% of that found for 10^5 untreated cells, and the CFU-S recovery was 7.0% ± 2.6% or 1.9 ± 0.5 actual spleen colonies. The mean number of progenitors observed from all of the experiments was 101 ± 4 CFU-GM and 28 ± 6 CFU-S for 10^5 untreated marrow cells assayed.

To verify that the hematopoietic reconstitution following transplantation with 5 x 10^5 bone marrow cells was actually of donor origin, lethally irradiated female B6D2f1 mice were transplanted with 5 x 10^5 marrow cells from male B6D2f1 mice. A total of 1/6 mice from three separate experiments survived. Two additional mice from each experiment were sacrificed at day 21 after transplantation for Y chromosome analysis of bone marrow by the Southern blot analysis of DNA, using a Y-specific DNA fragment.21 All mice appeared to be completely reconstituted by donor-derived cells. Southern blots from three of these mice are shown in Fig 1. In addition, two female mice were sacrificed for DNA analysis of bone marrow cells 9 months after BMT with 5 x 10^5 male bone marrow cells. Bone marrow from both mice still appeared to be completely donor derived.

Studies of the hematopoietic function of murine syngeneic marrow grafts after in vitro incubation with 4HC, vincristine, bleomycin, and 5FU were then performed. Graded doses of the four drugs were incubated with 10^5 bone marrow cells at 25°C for one hour. Figure 2 shows the CFU-GM and CFU-S results. The fractional recoveries (log) for CFU-GM and CFU-S are again similar, and both are significantly linearly related to the dose of 4HC, vincristine, or bleomycin. There was no difference in the numbers of spleen colonies recovered on day 7 or 12 from marrow incubated with graded

<table>
<thead>
<tr>
<th>No. of Marrow Cells</th>
<th>10^4</th>
<th>5 x 10^4</th>
<th>10^5</th>
<th>5 x 10^6</th>
<th>10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM recovery (%)</td>
<td>1.2 ± 0.7</td>
<td>5.4 ± 0.6</td>
<td>9.8 ± 0.5</td>
<td>54.3 ± 1.9</td>
<td>100 ± 1.7</td>
</tr>
<tr>
<td>CFU-S recovery (%)</td>
<td>0</td>
<td>7.0 ± 2.6</td>
<td>10.4 ± 1.2</td>
<td>60.4 ± 5.4</td>
<td>100 ± 3.2</td>
</tr>
<tr>
<td>No. of CFU-S observed</td>
<td>0</td>
<td>1.9 ± 0.5</td>
<td>2.9 ± 0.6</td>
<td>16.9 ± 2.4</td>
<td>28 ± 0.6</td>
</tr>
<tr>
<td>Survival of mice</td>
<td>0/8</td>
<td>14/16</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. The number of CFU obtained from 10^5 cells was taken as 100%.
doses of these three drugs. However, there were many more spleen colonies seen on day 12 than on day 7, with bone marrow incubated with 5FU. In fact, no spleen colonies were obtained on day 7 from 10^6 marrow cells treated with doses of 5FU greater than 60 µg/mL, despite getting an 8.7% ± 0.9% and a 6.0% ± 0.8% recovery of day 12 CFU-S with 5FU doses of 80 µg/mL and 100 µg/mL respectively. Figure 2 D shows that the same significant linear relationship exists between CFU-GM, day 12 CFU-S, and the in vitro incubation dose of 5FU, as is seen with the other three drugs. No increase in moderate-to-large-sized spleen colonies was seen after day 12 for untreated marrow or marrow treated with any of the four drugs. However, a "second wave" of many minute but visible spleen colonies appeared on days 13 to 14 with all syngeneic marrow transplants, as others have described.25 Table 2 shows that the highest incubation doses of drugs that allowed predictable engraftment by 10^7 marrow cells were again associated with approximately a 5% recovery of both CFU-GM and CFU-S or a recovery of one to two spleen colonies. This is the same recovery seen with assaying 5 x 10^7 untreated marrow cells.

4HC incubations under the above conditions were also performed using 10^6 and 10^7 marrow cells. Results are shown in Table 3. The highest incubation dose of 4HC that allowed survival of the lethally irradiated mice was 10 µg/mL for 10^6 marrow cells and 80 µg/mL for 10^7 cells. The former condition produced a 48% ± 6% recovery of CFU-GM and a 36% ± 1% recovery of CFU-S. The latter condition produced a 0.5% recovery for both progenitors. Both of these conditions produced one to two spleen colonies. The percent recoveries of CFU-GM and CFU-S for drug-treated bone marrow were independent of the number of marrow cells assayed. Engraftment of lethally irradiated mice by syngeneic marrow incubated with drugs was directly related to the number of transplanted CFU-GM or CFU-S. This equals the product of the pretreatment bone marrow CFU-GM or CFU-S content and their fractional recoveries after treatment. Again, survival of the mice occurred when the CFU-GM or CFU-S content of the transplant equalled that of 5 x 10^3 untreated bone marrow cells. Combining the survival results from all the different conditions tested (Tables 1, 2, and 3) reveals that 132 mice received transplants that produced at least one spleen colony. Of these 132 mice, 124 survived. Transplants producing less than one spleen colony allowed only 1% mice to survive.

To assess the kinetics of hematologic recovery, neutrophil counts on days 7, 10, and 14 after syngeneic BMT were performed. Figure 3 shows the circulating neutrophil counts on days 7, 10, and 14 after marrow transplantation with graded doses of untreated bone marrow. Mice receiving at least 10^6 marrow cells had normal neutrophil counts (greater than 2,000 absolute neutrophils) by day 14, and all surviving mice had normal neutrophil counts by day 21. The speed of neutrophil count recovery increased with increasing the number of transplanted cells up to a maximum of 5 x 10^6 cells. There were no differences in the recovery kinetics for marrow inoculums of 5 x 10^5, 10^6, or 2 x 10^6 cells. All mice developing 100 or more neutrophils/µL by day 10 survived. Interestingly, even mice transplanted with 10^6 marrow cells developed a small number of circulating neutrophils by day 10, although all these mice eventually died. No radiation control mice developed circulating neutrophils. Table 4 shows the results from periodic sampling of circulating neutrophil counts after transplanting 10^6 marrow cells that had been incubated in vitro with graded doses of 4HC. Untreated bone marrow transplants and 4HC-treated transplants, which contained similar numbers of CFU-GM or CFU-S, produced similar neutrophil recoveries. RBC recovery was parallel to neutrophil recovery for all of the conditions studied (data not shown). However, the nadir and ultimate recovery of the RBC counts lagged slightly behind the neutrophil counts.

DISCUSSION

Although CFU-GM assays have been used previously to study the effects of potential "purging agents" on the hematopoietic function of bone marrow,3,9,12,13,14 there was no clear evidence that this assay correlated with hematopoietic recovery. While some series of patients receiving "unpurged" BMTs have suggested a relationship between the number of CFU-GM infused and the speed of hematopoietic recovery,26-29 others have not.30,31 Earlier reports from this institution also indicated that the CFU-GM assay could not be used to predict hematologic recovery after 4HC "purged" autologous marrow transplantation, since marrows treated with 100 µg/mL of 4HC allowed engraftment despite no recovery of CFU-GM.32 We have subsequently found that the hematologic recovery of lethally irradiated mice receiving BMTs was directly related to the number of CFU-GM or CFU-S transplanted. Untreated and drug-treated bone marrow grafts with similar CFU-GM and CFU-S content produced similar hematologic recovery in transplanted mice, despite there being large differences in the numbers of transplanted...
Fig 2. Relation of percent survival of CFU-GM (O—O) and CFU-S (•—•) for 10^5 marrow cells incubated in vitro with 4HC (A), vincristine (B), bleomycin (C), and 5FU (D). (A through C) CFU-S results are the same on both days 7 and 12. (D) Only day 12 CFU-S are shown (see text). Each data point represents the mean ± SEM of two to four experiments performed in quadruplicate. Pearson's correlation coefficients (r) for all linear regressions are significant (P < 10^-4).

Table 2. CFU-GM and CFU-S Recovery With Survival of Mice After Transplantation With 10^5 Bone Marrow Cells Incubated With Graded Concentrations of Drugs

<table>
<thead>
<tr>
<th>Drug Concentration</th>
<th>CFU-GM-recovery (%)</th>
<th>CFU-S-recovery (%)</th>
<th>No. of CFU-S observed</th>
<th>Survival of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HC(μg/mL)</td>
<td>Vincristine (μg/mL)</td>
<td>5FU(μg/mL)</td>
<td>Bleomycin (mU/mL)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>5.1 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>140</td>
<td>5.2 ± 0.9</td>
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<tr>
<td>80</td>
<td>5.4 ± 1.3</td>
<td>1.9 ± 2.6</td>
<td>160</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>100</td>
<td>6.0 ± 0.8</td>
<td>2.1 ± 0.5</td>
<td>120</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>120</td>
<td>6.0 ± 0.8</td>
<td>2.1 ± 0.5</td>
<td>800</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>1200</td>
<td>9.7 ± 0.9</td>
<td>1.8 ± 0.8</td>
<td>200</td>
<td>3.7 ± 0.8</td>
</tr>
</tbody>
</table>

CFU-S results are the same on both day 7 or 12 for 4HC, vincristine, and bleomycin. CFU-S results with 5FU are only for day 12. Data are expressed as mean ± SEM.
drug incubation. Similar culture conditions with human marrow, as were used in previous reports of “purging” with 4HC from this institution, however, permitted only a 5% to 10% CFU-GM recovery to be seen. Nutrient enrichment in our agar culture using 30% FCS, 1% bovine serum albumin, and 10⁻⁴ mol/L 2-mercaptoethanol has allowed us to see nearly a tenfold increase in CFU-GM cloning efficiency without a change in CFU-GM drug sensitivity (unpublished data). We are now able to culture CFU-GM from all marrow “purged” with 100 μg/mL of 4HC. Utilizing similar enriched culture conditions, Rowley et al were able to find an average CFU-GM recovery of 4.5% (range 0.07% to 23%) after “purging” autologous marrow grafts with 100 μg/mL of 4HC. These much smaller fractional recoveries of CFU-GM permitted us to establish the correlation between CFU-GM and hematopoietic recovery, while the previously used culture conditions could not.

Second, there appears to be a maximum number of transplanted progenitors that allows the correlation with speed of hematologic recovery to be seen. We found no detectable difference in the kinetics of hematopoietic recovery in mice receiving $5 \times 10^6$ untreated marrow cells or greater, although the kinetics of hematologic recovery were strongly correlated with the number of cells or CFU-GM transplanted below this level. The CFU-GM content averaged over 2 to $10^5$ CFU-GM/kg, with all patients receiving at least $5 \times 10^4$ CFU-GM/kg, in the marrow transplants from the studies that showed no correlation between CFU-GM and hematopoietic reconstitution. Conversely, in four of the studies showing a relationship, an average of only $3 \times 10^4$ to $4 \times 10^4$ CFU-GM/kg were transplanted per patient. Our present animal data and the results of these clinical studies taken collectively suggest that there exists a maximum value for the number of hematopoietic progenitors transplanted, above which the rate of hematologic recovery following BMT is rapid and no detectable increase in the rate can be seen with increased CFU-GM content. However, below this maximum value hematopoietic recovery correlates with the CFU-GM content of transplanted marrow.

Hematopoietic reconstitution occurred in most mice receiving a bone marrow graft containing at least $5 \times 10^3$ untreated bone marrow cells. This is consistent with the findings of Boggs et al, who found that the inherited macrocytic anemia of W/W mice could be cured with $10 \times 10^3$ $+/+$ bone marrow cells. Mice appeared to require at least 100 circulating neutrophils/μL by day 10 after BMT to survive. Although all mice that received either $10^3$ untreated marrow cells or $10^2$ cells incubated with 80 μg/mL of 4HC died, they still developed some circulating neutrophils (but less than 100 μL) by day 10. This suggests that early engraftment may be developing but not quickly enough to prevent death from the complications of aplasia. Therefore hematopoietic reconstitution and survival in mice after BMT may be related to the speed of hematologic recovery. Although there was a clear minimum number of CFU-GM

<table>
<thead>
<tr>
<th>Drug Concentration (μg/mL)</th>
<th>10⁴</th>
<th>10³</th>
<th>10²</th>
<th>10¹</th>
<th>10⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM recovery (%)</td>
<td>48 ± 6.4</td>
<td>11 ± 5.0</td>
<td>5.1 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>0.54 ± 0.06</td>
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<tr>
<td>CFU-S recovery (%)</td>
<td>36 ± 1.0</td>
<td>0</td>
<td>5.4 ± 1.3</td>
<td>1.9 ± 2.6</td>
<td>0.51 ± 0.01</td>
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<tr>
<td>No. of CFU-S observed</td>
<td>1.1 ± 0.2</td>
<td>0</td>
<td>1.5 ± 0.4</td>
<td>0.6 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Survival of mice</td>
<td>7/8</td>
<td>2/8</td>
<td>10/10</td>
<td>0/10</td>
<td>7/8</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

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**Table 3. CFU-GM and CFU-S Recovery With Survival of Mice After Transplantation With Graded Concentrations of Bone Marrow Incubated With Graded Doses of 4HC**

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**Table 4. Circulating Neutrophil Counts/μL After Transplantation of $10^5$ Marrow Cells Incubated In Vitro with 4HC**

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Fig 3. Kinetics of circulating neutrophil recovery after transplantation of graded concentrations of untreated bone marrow cells. There were no differences in results for marrow transplants containing $5 \times 10^2$, $10^2$, or $2 \times 10^2$ cells. All mice transplanted with $5 \times 10^3$ or more cells survived. Those transplanted with $10^2$ cells died between days 10 and 14. Each data point represents the mean ± SEM of eight mice from two separate experiments.
or CFU-S needed to allow survival of the transplanted mice, it is possible that mice receiving much smaller numbers of progenitors could survive with increased support during aplasia.

We found no difference between the numbers of day 7 and day 12 CFU-S after transplantation with untreated marrow or marrow treated with 4HC, vincristine, or bleomycin. However, we did find that the numbers of day 12 spleen colonies were increased compared to day 7 after in vitro incubation of bone marrow with 5FU. Others have also reported that while the numbers of CFU-S remained constant between day 8 and day 14 after transplantation with untreated bone marrow, the numbers of CFU-S following transplantation with 5FU-treated marrow increased over this time.2-5 Hodgson and Bradley25 proposed that this increase in spleen colony numbers with time after transplantation of 5FU-treated marrow may be due to noncycling stem cells that are more resistant than committed progenitors to 5FU. However, we found that day 12 CFU-S have the same sensitivity to 5FU as the committed hematopoietic progenitor, CFU-GM. This finding may suggest that the delayed extra spleen colonies that appear after 5FU treatment occur as CFU-S repair sublethal damage done by 5FU, as was proposed by Rosendaal et al.26

We have found, as have others,35,36,37 that the CFU-GM and CFU-S show similar sensitivities to drugs. It has been reported that the cells that form both of these colonies are relatively mature progenitors and thus are not the cells responsible for marrow engraftment.35,37,38 Recent reports have also described a primitive, multipotential progenitor (the blast colony-forming cell) that is more resistant than CFU-GM to 4HC.39,40 These reports make it difficult to explain the ability of CFU-GM and CFU-S to predict hematopoietic reconstitution after BMT. One possibility is that although the CFU-GM and CFU-S are not the cells responsible for engraftment, they could still predict the response of the stem cell to manipulations if their quantities and drug sensitivities remained proportional to those of the stem cell. Evidence against this hypothesis is that reconstitution in our study was related to the same degree of CFU-GM inhibition after in vitro incubation of the marrow with four different drugs. It is unlikely that drug sensitivity differences between the stem cell and CFU-GM would be the same for all four drugs. In fact, Sahovic et al.41 showed that although the primitive multipotential progenitors were more resistant than CFU-GM to 4HC, there were no differences in sensitivities to the alkylating agent, phenyl-keto-phosphamide.41 A more likely explanation for the ability of CFU-GM and CFU-S to correlate with hematopoietic reconstitution is that there may be two phases of engraftment after BMT. Early hematopoietic recovery could be due to mature committed progenitors, while primitive pluripotential progenitors would be responsible for later, sustained engraftment. If this were the case, initial engraftment would be expected to correlate directly with committed progenitors like the CFU-GM.

Both the CFU-GM and CFU-S content of bone marrow, therefore, predict hematopoietic recovery after syngeneic transplantation in mice. This is true even for marrow treated with drugs like 4HC, which may have differential activity toward committed and multipotential hematopoietic progenitors. A minimum number of transplanted CFU-GM or CFU-S is needed to allow survival of lethally irradiated mice. There is also a maximum number of transplanted progenitors above which the correlation between progenitor content in the transplant and speed of hematopoietic recovery is not seen. Obviously the true effectiveness of any purging regimen for autologous BMT can only be assessed from clinical trials. However, the CFU-GM assay should prove to be a useful technique to assess the effect of potential "purging" agents on hematopoietic function.

REFERENCES


Progenitor cell assays predict hematopoietic reconstitution after syngeneic transplantation in mice

RJ Jones, SJ Sharkis, P Celano, OM Colvin, SD Rowley and LL Sensenbrenner