A Primary Stem Cell Lesion in Experimental Chronic Hypoplastic Marrow Failure

By Alec Morley, Kevin Trainor, and Judith Blake

Following a course of busulfan, mice sustain a residual injury to the marrow which is characterized by prolonged or permanent mild marrow hypoplasia and which frequently terminates in severe marrow aplasia and death. The stem cells and the marrow environment of mice bearing this residual injury were studied by assessing the ability of cells from these mice to grow when transplanted into irradiated normal hosts and by assessing the ability of normal cells to correct the residual injury when transplanted into mice previously treated with busulfan. Stem cells from busulfan-treated mice grew poorly, both in the tibial and splenic environment of normal recipients. Normal cells transplanted into busulfan-treated mice corrected most of the lesion in that they restored the number of granulocytic progenitor cells to normal, the number of pluripotential stem cells to, or almost to, normal; but they were unable to restore completely the number of more differentiated, nucleated marrow cells. Thus, the major factor in residual lesions produced in mice by busulfan is a lesion of the stem cell itself, but an additional minor factor may be a lesion of the marrow environment.

RECENTLY WE REPORTED that administration of busulfan to mice resulted in prolonged or permanent residual injury to the bone marrow and that the latter was followed in many mice by frank marrow failure, pancytopenia, and death. The stage of residual injury was characterized by little or no change in the peripheral blood, a mild decrease in marrow cellularity, and a more marked decrease in the number of colony-forming units (CFU) and colony-forming cells (CFC) in the marrow. This failure to restore normal numbers of marrow cells could be due either to an intrinsic lesion of the cells themselves and/or to a lesion of the environment in the animal. We have studied these possibilities by investigating the ability of the cells from busulfan-treated mice to proliferate and differentiate in normal irradiated hosts and by determining the ability of normal marrow cells to repair the residual lesion when transplanted into busulfan-treated mice.

MATERIALS AND METHODS

Randomly bred, virgin female Balb/C mice, 10–16 wk of age, were obtained from the Institute of Medical and Veterinary Science, Adelaide, and given food and tap water ad libitum. To the water was added 1 g neomycin and 1 ml of 1% sodium hypochlorite per liter. Busulfan was administered as previously described in doses of 20, 20, 20, and 10 mg/kg intraperitoneally at

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intervals of 2 wk. Following the final injection, the mice were maintained for at least 2 mo before being studied. Control mice were mice of the same age which had not been injected.

Mice 10–16 wk of age were used as a source of normal marrow cells and as recipients in transplantation experiments.

**Assays**

Tibial cell counts were made by cutting off the ends of one tibia, expressing the cells into saline, suspending them, and counting the resultant suspension. Differential counts were made on 200–250 cells in smears of femoral marrow. CFC were assayed essentially by the method of Bradley and Metcalf. Cells at a concentration of $5 \times 10^5$ were grown for 7–8 days in soft agar in modified Eagles medium at 36.5°C in an atmosphere of 7.5% CO$_2$ and 4% O$_2$ in N$_2$ at 100% humidity and were stimulated by an extract of pregnant-mouse uterus. CFU were assayed by the method of Till and McCulloch in which marrow cells pooled from four to five donor mice were injected into eight to ten recipient mice which had been irradiated 1–2 hr previously with 650 rads (250 kV; 12 mA; 102 rpm; no added filtration; HVL, 0.7 mm of Cu). The irradiated mice received 2 g streptomycin, 2 g tetracycline, 2 g sodium sulfamethazine, and 2 ml of 1% sodium hypochlorite per liter of drinking water. Spleen colonies were counted 8 days later. Under these conditions, uninjected irradiated mice produced a mean of 0.29 spleen colonies (determined from 129 mice) and had a survival of 82% at 24 days after irradiation.

**Experimental Design**

The normality of the cells from busulfan-treated mice was examined in three experiments. In each of these experiments, irradiated normal mice either were transplanted with normal marrow cells or with cells from busulfan-treated mice, or were not transplanted. An attempt was made to transplant approximately equal numbers of CFU into the first two groups. On days 8, 12, 16, 20, and 24, four to five mice from each group were sacrificed, a tibial nucleated cell count was performed on one of the tibias, and the cells from the remaining tibias were pooled for CFU and CFC assays. In the final analysis of the results, all of the values from the three experiments were logarithmically transformed, pooled, and means and standard errors for each time point calculated.

In three experiments, the ability of the cells from busulfan-treated and normal mice to self-renew and differentiate in the spleen was studied by investigating the ability of splenic CFU to form further CFU and CFC. This was done using the whole-spleen method. Low doses of marrow cells were transplanted into irradiated normal recipients, and the number of colonies in the spleen on day 9 was determined. Tenfold greater doses of cells were injected into further groups, and five animals from each group were sacrificed on day 9. Single cell suspensions were made by forcing minced splenic tissue through a stainless steel filter and then drawing the resultant material through a needle. The numbers of CFU and CFC in these suspensions were determined by retransplantation and tissue culture of appropriate aliquots. The numbers of CFU and CFC per spleen colony, that is, arising from one CFU, were calculated from the results. Owing to technical problems, it was not possible to obtain the number of CFC per colony in one of the three experiments.

In two experiments, the normality of the marrow environment was tested in mice which had received busulfan in a course ending 2 mo previously. In one experiment $9.2 \times 10^5$ normal cells were transplanted into busulfan-treated mice and in the other $13.9 \times 10^6$ cells were transplanted. Nontransplanted busulfan-treated mice from the same batch and normal mice of the same age were used as controls. At various times ranging from 19 to 96 days after transplantation, the tibias of groups of four to five mice from each batch were assayed for their content of CFU, CFC, and nucleated cells. Two assays were performed during the course of the first experiment and four were performed during the course of the second. In three assays, femoral smears were also made and the numbers of erythroid, granulocytic, and lymphocytic cells in a total of 200–250 nucleated cells were counted. The results of all assays were logarithmically transformed, and the significance of any differences found between groups was tested by performing paired t-tests.
Table 1. Cells Transplanted for Growth Curves

<table>
<thead>
<tr>
<th></th>
<th>CFU</th>
<th>Nucleated Cells</th>
</tr>
</thead>
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<tr>
<td>Expt 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Busulfan</td>
<td>580</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>Control</td>
<td>230</td>
<td>$0.7 \times 10^6$</td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Busulfan</td>
<td>120</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Expt 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Busulfan</td>
<td>100</td>
<td>$2.5 \times 10^6$</td>
</tr>
<tr>
<td>Control</td>
<td>180</td>
<td>$0.5 \times 10^6$</td>
</tr>
</tbody>
</table>

RESULTS

Table 1 shows the numbers of CFU and nucleated cells transplanted in the three experiments in which growth curves in the tibia were studied. Despite the differences in numbers of cells transplanted, the results were identical. The pooled findings are shown in Fig. 1. The CFU and CFC from busulfan-treated mice showed a clear difference from those in the control cells in that they manifested a delay of approximately 4 days in the onset of self-renewal, a slower rate of self-renewal in the logarithmic phase of growth, and a lower final plateau in cell number. However, the results of the growth curves in irradiated...
mice which had not received any cells indicated that most of the apparent growth in the busulfan-treated group was in fact not due to the busulfan-treated cells but to residual surviving endogenous hemopoietic cells. Since the results on the ordinate shown in Fig. 1 are expressed on a logarithmic scale, subtraction of the recovery curve for endogenous CFU from the curve obtained for control cells would have little effect on the exponential phase of the latter, whereas subtraction from the curve for busulfan-treated cells would result in a greatly decreased slope of recovery. Thus, the difference in the growth curves of normal and busulfan-treated cells grossly underestimated the true difference in self-renewal ability between the two types of cells.

Table 2 shows the results of the three experiments comparing the growth of normal and busulfan-treated CFU in the spleen. Colonies derived from normal cells contained 3–13 times as many CFU and 15–21 times as many CFC as did those derived from busulfan-treated cells. Thus, busulfan-treated CFU were grossly defective in their ability to produce further CFU. Superficially, as judged by the CFC/CFU ratios, the CFU in colonies derived from busulfan-treated cells were only slightly defective in their ability to proliferate during differentiation to CFC. However, since CFC at any one time are derived, not from CFU present at the same time, but from CFU which differentiated somewhat earlier, the denominator of the CFC/CFU ratio should be the number of CFU present at that earlier point in time. When one corrects for the greatly different growth rates of normal and busulfan-treated CFU, the number of normal CFU at that time would have been much less, whereas the number of busulfan-treated CFU would have been little different. Thus, the corrected CFC/CFU ratio was undoubtedly much greater for normal cells, indicating that CFU from busulfan-treated mice were also markedly defective in their ability to produce CFC.

Table 2. Self-renewal and Differentiation of CFU in the Spleen

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CFU per Colony</th>
<th>Control Busulfan</th>
<th>CFC per Colony</th>
<th>Control Busulfan</th>
<th>CFC/CFU</th>
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</thead>
<tbody>
<tr>
<td>One</td>
<td>0.43</td>
<td>13.3</td>
<td>1.01</td>
<td>21.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Control</td>
<td>5.58</td>
<td>21.4</td>
<td>3.0</td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>Two</td>
<td>0.32</td>
<td>3.0</td>
<td>0.22</td>
<td>15.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.97</td>
<td>12.9</td>
<td>3.38</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
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The results obtained from the experiments involving transplantation of normal cells into busulfan-treated mice are shown in Fig. 2. Normal cells raised the mean number of CFU from 16% to 72% of the mean control value; this effect was highly significant (p < 0.0025), but the failure completely to correct
Fig. 2. Numbers of CFU, CFC, and nucleated cells in busulfan-treated mice, busulfan-treated mice transplanted with normal cells, and in control mice. Each point represents a result from four to five tibias.

The defect was also perhaps significant ($p < 0.05$). Normal cells raised the mean number of CFC from $6\%$ to $92\%$ of the mean control value; this effect was also highly significant ($p < 0.0005$), but the final value did not differ significantly from the control ($p > 0.4$). Normal cells increased the number of nucleated cells per tibia from $43\%$ to $76\%$ of the mean control value; this effect was highly significant ($p < 0.0005$), but the failure to raise this number completely was equally significant. As indicated in Fig. 3, the persisting deficit of nucleated cells involved all of the major cell types—erythroid, granulocytic, and lymphocytic—rather than any one particular cell type.

DISCUSSION

The results involving transplantation of busulfan-treated cells into normal hosts clearly indicate that the CFU from busulfan-treated mice are grossly defective in their ability to self-renew and in their ability to differentiate and produce CFC. The abnormality of self-renewal is manifest during proliferation in both tibia and spleen. Whether the defect primarily involves the CFU or primarily involves another cell which interacts with the CFU is a subject for further investigation. The ability of normal marrow cells to correct most of the defect of busulfan-treated mice indirectly confirms that the CFU are abnormal, and directly indicates that, in large part, the environment in busulfan-treated mice is normal. Nevertheless, the inability of normal cells to correct the defect completely may indicate that an environmental lesion plays a minor role. An alternative explanation, however, is that the proliferative demands placed on normal cells as they repair the defect leads to some loss in proliferative ability.
There is some evidence that such a phenomenon can occur. Whether the residual injury to stem cells of busulfan-treated mice is due to a direct effect of busulfan, or to the enhanced demands for proliferation consequent on hemopoietic depletion produced by busulfan, is a question about which one can only speculate. The residual injury in our mice resembles the residual injury produced after repeated irradiation. The results of most workers who have studied this phenomenon suggest that residual injury following irradiation is due to stem cell impairment occurring as a consequence of the sustained demand for proliferation incurred by the hemopoietic cells. This interpretation is, however, not completely accepted, since Hendry and Lajtha have claimed that the injury is due to a lesion of the marrow environment. Be that as it may, our results indicate that the latter factor cannot be of prime importance in busulfan-treated mice.

In view of the marked loss of proliferative potential of the CFU in busulfan-treated mice, it is tempting to suggest that the final marrow failure which occurs in a proportion of these mice is due to a final exhaustion of the proliferative potential of the CFU, analogous to the Hayflick effect which occurs in vitro. However, our results do not provide any direct evidence on this point, and other possibilities must be considered. Such possibilities might include exposure to a further marrow toxin, perhaps one which would be relatively innocuous in normal mice, infection with an exogenously acquired aplasia-producing virus (analogous to infection with the virus of infectious hepatitis in man), or, possibly, activation of an endogenous aplasia-producing virus.

If the experimental situation produced by busulfan treatment of mice is a valid animal model of aplastic anemia in man, then the finding of a prolonged impairment of self-renewal of stem cells would account for some of the features observed in the human disease. It would provide an explanation for the pro-
longed course of the disease, and for the ability of transplanted, immunologically compatible cells to produce a remission.13

Few investigations appear to have been performed into the question of residual injury following cytotoxic drugs, but such studies, although limited to the effect of cytotoxic drugs on L1210 leukemia cells, also seem to suggest that residual injury may occur.14–18 Cytotoxic agents are usually regarded as causing quantitative changes in hemopoietic cells as the result of direct cell killing, whereas the phenomenon discussed in the present paper is a different type of disturbance in that it is a qualitative impairment of the ability of normal stem cells to self-renew. Such a property of cytotoxic drugs would be of importance with regard to cancer chemotherapy. Whether agents other than busulfan have the same effect is a question presently under active investigation.

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REFERENCES

17. Johnson RE, Hardy WG, Zelen M: Chemotherapeutic effects on mammalian tumor
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