Busulfan-Induced Chronic Bone Marrow Failure: Changes in Cortical Bone, Marrow Stromal Cells, and Adherent Cell Colonies

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This study represented an effort to determine if there were quantitative or morphological changes in marrow stromal cells in busulfan-induced marrow failure and to relate these changes to other disturbances in blood and bone marrow. Mice received four busulfan (BU) injections at two-week intervals and were killed at various time points up to 40 weeks after the first injection. Evaluation techniques included complete blood counts, in vitro assay of short-term adherent cell colonies per femur (STACC per femur) and colony-forming unit-culture (CFU-C) per femur, light microscopy of sternebral marrow and spleen, and electron microscopy (EM) of sternebral marrow taken at 40 weeks. STACC per femur were acutely reduced to 25% of control, but recovered to 76% by 40 weeks. CFU-C per femur dropped to below 10% of control and never recovered. Histologically, we found that hypoplasia of acutely affected marrow was associated with heightened endosteum and cortical bone thickening. In the chronic phase of BU injury, bones became osteoporotic, and the frequency of adipocytes and mast cells rose. BU-affected spleens generally had enhanced erythropoiesis. No stromal cell changes in 40-week marrow were discernable by EM. We concluded that there were morphological changes in BU marrow stroma specifically involving endosteum, bone, adipocytes, and mast cells. Also, there was quantitative depression in stromal cells measured by the STACC assay, but this improved substantially with time, unlike damage to hematopoietic stem cells measured by the CFU-C.

MORLEY AND BLAKE attributed the aplastic anemia induced in mice by multiple doses of busulfan (BU) to a proliferative defect in the hematopoietic stem cell. However, marrow transfusion cannot restore the splenic colony-forming unit (CFU-S) to levels greater than 72% of normal. Damage to the stroma, upon which expression of CFU-S depends, may therefore contribute to the marrow hypoplasia.

Fried et al., using their bone implant technique, showed that marrow stromal function declined by 50% after BU administration. Anderson et al² and Hayes et al³ found that marrow-derived adherent cells from BU-treated mice failed to support normal CFU-S and CFU-C.

These studies suggest that stromal damage may indeed contribute to BU-induced marrow failure. This study was undertaken to determine by tissue culture and light and electron microscopy the quantitative and morphological extent of this damage. Adherent cell precursors were assayed by culturing marrow and counting adherent cell colonies before confluence. We designated this the short-term adherent cell (STAC) culture, and the number of STAC colonies per femur (STACC per femur) was assayed. We adopted the term STACC rather than CFU-F (as used by others⁴) because cells in these cultures were heterogeneous rather than purely fibroblastic.

MATERIALS AND METHODS

Animals and Treatment

Virgin female A/J mice (Jackson Laboratories, Bar Harbor, Me) were housed in filter-top cages and fed and watered ad libitum. Water was treated with neomycin (1 g/L). Fifty milligrams pure busulfan (Burroughs Wellcome, Research Triangle Park, NC) was dissolved in 5 mL acetone, which was further diluted with 20 mL bacteriostatic water to yield 2 mg/mL.

Eight- to 10-week-old mice were injected intraperitoneally on days 0, 14, 28, and 42. Weighed to the nearest 0.5 g, they received 20 mg/kg BU for each of the first three injections and 10 mg/kg for the fourth. Controls received comparable volumes of acetone and water.

Mice were killed on days 3, 7, 10, 14, 21, 28, 35, 42, 49, 56, 112 (week 16), 210 (week 30), and 280 (week 40). Total and differential WBC counts, hematocrits, and marrow myeloid to erythroid ratios (M:E) were determined for each animal. Femoral marrow provided cells for both M:E and cultures on days 56, 112, 210, and 280. Spleen and sternebrae were taken for light microscopy and sternebrae for electron microscopy.

Peripheral Blood

Under ether anesthesia, approximately 0.3 mL blood was collected from the retroorbital sinus using heparinized capillary tubes. WBC counts were determined with a Coulter ZBI cell counter (Coulter Electronics, Hialeah, Fla). Hematocrits were measured from spun microhematocrit tubes. Smears for white cell differentials were stained with Diff-Quik (American Scientific Products, McGraw Park, III).

Myeloid:Erythroid Ratios

M:E ratios were determined after counting 500 or more cells on Giemsa-stained smears of either femoral marrow fragments or the pellet made by centrifugation of the marrow cell suspensions used for culture.

Cultures

Each femur was removed aseptically from a freshly killed mouse. Ends were snipped off and marrow flushed out with 1.0 mL.
alpha-minimal essential media (α-MEM; Gibco, Grand Island, NY) per femur. Single-cell suspensions were made by thorough mixing and multiple passages through increasingly smaller gauge needles. Counts were expressed as the number of nucleated cells per femur. For short-term adherent cell (STAC) cultures, marrow cells were suspended at 0.5 × 10⁶ cells/mL α-MEM, supplemented with 25% fetal calf serum, penicillin, and streptomycin. Four 35 × 10 mm Petri dishes per mouse were plated with 2 mL of media containing 10⁶ cells and placed in a Forma water-jacketed incubator at 37 °C, 5% CO₂. After eight days, dishes were drained of media. Adherent cells were fixed in absolute methanol for five minutes, dried, and stained with Giemsa for 45 minutes. Colonies (each defined as having at least 30 cells) were counted with a Wild dissecting microscope at 12 to 25× and were expressed as the number of colonies per 10⁶ cells. A mean was calculated for the four Petri dishes. The nucleated cell count per femur was used to determine the number of short-term adherent cell colonies per femur (STACC per femur).

Following Metcalf’s procedure for soft agar gel culture (CFU-C), marrow cells were suspended in media at 10⁶ cells/mL. One milliliter was inoculated per Petri dish (35 × 10 mm) and four dishes per mouse were cultured. Endotoxin-stimulated mouse serum, diluted 1:6 with water, was the source of colony-stimulating factor. Each Petri dish received 0.1 mL of this diluted serum just before inoculation with marrow suspension. The suspension was allowed to gel at room temperature before incubating at 37 °C and 10% CO₂ for seven days. Colonies (each defined as having at least 50 cells) were counted on a dissecting microscope at 12 to 25×. CFU-C per femur was determined using the same method described for STACC per femur.

For STACC and CFU-C cultures at 56 days, cell suspensions of three mice were pooled because the marrows of treated mice were too hypocellular to obtain sufficient cells from individual mice. Controls were handled in the same way.

Light Microscopy

Sternebrae and spleen were fixed in phosphate-buffered formalin for at least one week. Bones were then decalcified for two days in 10% formic acid with one change at 24 hours. Tissues were embedded in JB 4 methacrylate embedding media (Polysciences Inc, Warrington, Pa), sectioned at 1 μm with a Sorvall JB 4 microtome, and stained with May-Grünwald-Giemsa stains. Forty-eight controls and 67 experimental tissues were examined over ten months, beginning three days after the first BU injection. Using an ocular micrometer, the thinnest and thickest areas of cortical bone were measured for each animal.

Electron Microscopy

Sternebrae were fixed overnight at 4 °C in fresh Karnovsky’s fixative, washed, and stored in 0.1 mol/L cacodylate buffer (CAC), pH 7.4, at 4 °C, and decalcified in EDTA/glutaraldehyde at 4 °C for eight days with a change of solution every two days. After a final washing in CAC, they were treated overnight with 1% osmium tetroxide, washed in CAC, dehydrated, embedded in epon-araldite (Polysciences Inc), sectioned on a Sorvall MT-2 Ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Zeiss 10 CA electron microscope.

Statistics

Values for all parameters are expressed as mean ± 1 SD. Statistical analysis was done using the Wilcoxon rank sum test and Kruskal-Wallis one-way analysis of variance.

RESULTS

Busulfan-treated mice never showed normal weight gain with maturation. At 40 weeks, they averaged 18 g compared with the controls’ 28 g.

Hematocrits were depressed throughout the study. The overall mean for controls was 46.6% ± 1.3% compared to a mean of 43.0% ± 3.5% for experimental animals, (P<.001). Neutrophil counts were significantly decreased by two weeks, but recovered to normal levels by 16 weeks. Lymphocyte counts dropped substantially, but never recovered, always being about 26% of the control mean.

Marrow Changes

Marrow cellularity for experimental animals was always less than controls, but did show some recovery with time (Fig 1). During the acute phase, the M:E ratios were low (marrows were predominately erythroïd), but by week 16, they had recovered fully. STACC per femur in BU-treated animals was never as depressed as CFU-C per femur; furthermore, STACC per femur recovered substantially in time, while CFU-C did not.

The distribution chart for STACC per femur (Fig 2) shows that at weeks 8 and 16, many treated animals
had values less than 20% of the control mean. At 30 weeks, eight of 20 mice remained less than 20% of controls. Although the distribution of results was dissimilar, there appeared to be no difference between control and experimental values according to the Wilcoxon rank sum test. By 40 weeks, although only a few animals showed STACC per femur less than 40% of controls, a statistically significant ($P < .033$) difference was found between control and experimental values. The mean for BU-treated animals was 76% of the control mean.

**Light Microscopic Observations**

**Controls.** Sternebral marrow was normally packed with hematopoietic cells (Fig 3A). Myeloid cells were slightly more abundant than erythroid cells. Adipocytes and mast cells were rare. Normal cortical endosteum was consistently flat, but trabecular endosteum varied in thickness. Therefore, all comparisons between BU-treated animals and controls were based on examination of cortical endosteum. The shaft of bone had a cortical thickness that ranged from 40 to 90 $\mu$m at its thinnest and thickest parts. Blood vessels, other than the central vein, were rarely dilated; they accommodated approximately one to three RBC within their diameter. In normal spleen, white pulp, which dominated the core, was surrounded by a narrow subcapsular zone of red pulp. Typically, a few erythroblasts, mast cells, and megakaryocytes lay in red pulp.

**BU-treated, days 3 through 14.** In the two weeks following the first BU injection, the density of nucleated cells decreased slightly. The predominant cell type shifted from myeloid to erythroid. Some marrows lacked megakaryocytes, but most had them. The levels of mast cells and fat cells were not increased. Cortical endosteum and bone appeared normal, as did spleen.

**BU-treated, days 21 through 49.** The concentration of nucleated cells in these marrows was considerably reduced, with rare myeloid cells and only occa-

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Fig 3. (A) Normal sternebral marrow and cortical bone (190x). (B) BU-treated sternebral marrow, five weeks. The arrows in 3A and 3B point to endosteum. Adipocytes (a) were common in treated marrow (475x). (C) BU-treated cortical bone and erythroid marrow, eight weeks. Figs 3A and 3C were taken and printed at the same magnification. Note that the cortical bone in 3c is substantially thicker than control bone (190x). (D) BU-treated sternebral marrow and cortical bone, 30 weeks. This mouse had aplastic marrow and osteoporotic bone. Mast cells (MS) and adipocytes were abundant. Blood cells present were mostly mature (190x).
sional pockets of erythroid cells. No marrow contained megakaryocytes. Most marrows had increased numbers of adipocytes. Blood vessels in aplastic* areas were frequently disrupted. The major cell types remaining included macrophages, reticular cells, endothelial cells, RBCs, and fat cells. Hypocellular marrow, in general, had high cortical endosteum (Fig 3B). By day 49, occasional mice had an increase in the thickness of cortical bone. Despite aplastic marrows, most spleens had a widening of the red pulp due to increased levels of erythropoiesis. In some mice, there were megakaryocytes in the spleen, but none in the marrow.

**BU-treated, day 56.** In this group, cellularity increased in some animals and remained severely depressed in others. The morphology of those with decreased cellularity resembled the previous group. In all cases, erythroid cells outnumbered myeloid cells. The marrow of all mice showed substantial increases in the number of adipocytes and had occasional mast cells. Depression of megakaryocytopoiesis continued. Only one marrow contained megakaryocytes in normal numbers and size. Intact but dilated venous sinuses generally accommodated six to ten RBCs across their diameter. Cortical endosteum was high in areas with low cellularity. Cortical bone in all seven animals was substantially thicker than normal, ranging from 70 to 150 μm (Fig 3C). Spleens showed a marked increase in the number of erythroblasts, causing a widening of the subcapsular red pulp.

**BU-treated, days 112 and 210 (weeks 16 and 30).** Marrows from these animals showed substantial recovery. Although the density of the nucleated cells was less than in controls, all hematopoietic cells, including megakaryocytes, appeared normal. There was a marked increase in the numbers of fat cells and mast cells, the latter especially numerous at 30 weeks. For most mice, the diameter of venous sinuses had returned to normal, the endosteum was flat, and bone thickness normal. One 30-week mouse, however, had aplastic marrow and abnormal cortical bone. Its bone was uniformly thin and porotic (Fig 3D). Splenic erythropoiesis was no longer as intense, but was still enhanced relative to controls. In a few mice, mast cells were prevalent in red pulp. The aplastic mouse, in particular, had a markedly erythroid spleen with many mast cells.

**BU-treated, day 280 (40 weeks).** All mice discussed above had decreased STACC per femur. In this group, four of the nine mice examined by light microscopy (LM) had decreased STACC per femur, but the remaining five had normal STACC per femur. Light microscopy revealed no morphological differences between the two groups. All nine marrows had fair to good cellularity and hematopoietic cells of normal appearance. All had normal or only slightly decreased numbers of megakaryocytes. Slightly dilated blood vessels accommodated four to eight RBCs across their diameters. Mast cell and fat cell numbers were increased. Although the endosteum appeared normal, osteoporosis of the cortical bone, as seen in the 30-week aplastic mouse, was obvious in all mice. The range of bone thicknesses was 25 to 60 μm. Seven of the nine mice had erythroid spleens and many mast cells, with the latter confined to red pulp.

**Electron Microscopy of Sternum**

While the number of stromal cells (STACC per femur) were quantitatively normal at 40 weeks for many BU-treated mice, it seemed possible that observable qualitative changes in stromal cells might account for marrow depression. Therefore, the marrow of three 40-week controls and three 40-week experimentals with normal STACC per femur were studied by electron microscopy, with emphasis on adventitial reticular cells, macrophages, endothelial cells, and bone-lining cells. There were no differences between the two groups.

**DISCUSSION**

The quantitative assay, STACC per femur, was used because adherent cells are vital to hematopoiesis in vitro and are probably representative of the in vivo environment. The relevance of adherent cells to BU-induced marrow failure was indicated by Hays et al and Anderson et al, who showed that confluent adherent cells derived from BU-treated mice failed to support stem cells as well as adherent cells from normal mice. Hays et al showed that this loss in function persists at least 18 months after BU treatment.

In the acute phase, STACC per femur were significantly depressed, but with time, there was recovery to 75% to 80% of controls. This degree of recovery, although substantial, may set a limit on hematopoietic recovery even in the presence of normal hematopoietic cells. For example, Morley et al were unable to fully reconstitute hypoplastic mice with bone marrow transplants.

CFU-C per femur was more profoundly depressed than STACC and showed no recovery. There was, therefore, no correlation between STACC and CFU-C, indicating that these were separate, unrelated cell populations and that damage to these cell lines was

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*The term aplastic, as used in this article, refers to a marrow devoid of hematopoietic cells. Hypocellular or hypoplastic marrows contain hematopoietic cells, but in reduced numbers.
independent of each other. If depression of hematopoietic cells depended on stromal damage, some recovery in CFU-C would occur with recovery of STACC. Therefore, although stromal change may be a factor limiting hematopoietic recovery, it probably does not contribute to the development of marrow failure. More likely, busulfan affects stromal cells, as measured by the STACC assay, and hematopoietic cells independently and to different degrees. However, as indicated above, quantitative recovery does not assure functional recovery.

Despite the profound and lasting depression of CFU-C, circulating neutrophils in BU-treated animals were not significantly different from controls by 16 weeks. Moreover, Fitchen and Cline showed that BU-treated mice can generate an increase in granulocytic differentiation comparable to controls after stimulation by glucan. Boggs and Boggs suggested that marrow failure may occur because of an imbalance between stem cell differentiation and replication. Such an imbalance should cause a gradual decline in stem cells, but in our study, CFU-C remained at the same low level despite normal peripheral neutrophil counts.

Adipocytes were seen in increased frequency in BU-treated marrow, but no association between the number of adipocytes and STACC per femur was found. However, this is not proof that cells assayed via STACC cultures are not precursors to adipocytes. Fat cells are postmitotic, while STACC cultures assay cells with proliferative capacity. Normal neutrophil counts in BU-treated mice did not suggest the paucity of their precursors, the CFU-C. The same may be true for fat cells and STACC. These examples indicate that histology of the marrow and study of the peripheral blood may provide no clues as to the status of stem cells.

Acutely aplastic marrow apparently stimulated endosteal activity, resulting in cortical bone thickening. Chronically affected bone, however, was consistently osteoporotic. While these changes may have been due to direct injury, we believe they more likely represented a secondary response to other local or systemic factors.

Local factors appeared to induce the high endosteum seen in acute failure, since in sternebrae that had cellular marrow adjacent to hypocellular marrow, endosteum was high on the hypocellular side. Endosteum may contain more than bone progenitor cells and osteoclasts. Some bone-lining cells may be multifunctional and may participate in the regulation of hematopoiesis. Bone-lining cells secrete more colony-stimulating factor (CSF) than cells within the marrow. Furthermore, evidence indicates that CFU-S are concentrated close to bone. Shackney demonstrated a kinetic gradient across marrow, with the highest proliferative rate near bone. In their study of the early repopulation of marrow following irradiation, Lamber and Weiss found that undifferentiated and immature myelopoietic colonies favored subendosteal locations, while erythropoieses occurred away from bone. As regulators of hematopoiesis, bone-lining cells would be sensitive to changes in marrow homeostasis. In our studies, bone-lining cells may be hyperactive in the acute phase because of the lack of inhibitory feedback from their target cells, ie, hematopoietic cells.

In the later months of our study, cortical bone thinned and became porous. Mast cells, common in these marrows, secrete heparin, which may enhance the effects of parathyroid hormone and is associated with increased collagenase activity. In senile osteoporosis, an increase in mast cells often occurs in bone marrow. Furthermore, a side effect of chronic high-dose heparin therapy is osteoporosis.

It is also possible that because we used female mice, they may have suffered BU-induced ovarian suppression. Postmenopausal osteoporosis is common in women, although the pathogenesis is poorly understood. The same mechanism may be at work here, but these mice were not tested for fertility.

In sum, there seem to be two bone reactions. The first is an acute reaction to local factors in a marrow that is disrupted and aplastic. The second is a chronic general response with less obvious direct cause.

Mice with hypoplastic marrows generally had markedly erythroid spleens. Extramedullary hematopoiesis increases when medullary hematopoiesis is inadequate, as in chronic anemia or marrow replacement by malignant cells. In our study, there was a mild chronic anemia. These mice all had erythroid spleens, hypoplastic marrow, and reduced STACC per femur. Perhaps stromal disruption in these marrows was so severe that adequate erythropoiesis could not be supported, forcing this activity on the spleen. These observations suggest that BU-induced injury to stromal cells was more severe in the marrow than in the spleen.

In conclusion, BU apparently damages hematopoietic stromal cells, but quantitatively, this damage is not as severe as that in hematopoietic stem cells. This stromal cell defect may account for the failure of Morley and his colleagues to reconstitute aplastic mice with marrow transplants. Furthermore, bone-lining cells appear responsive to marrow aplasia, supporting the theory that they may be a link in the hematopoietic regulatory chain.

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Busulfan-induced chronic bone marrow failure: changes in cortical bone, marrow stromal cells, and adherent cell colonies

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