An Animal Model of Chronic Aplastic Marrow Failure. I. Late Marrow Failure After Busulfan

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An animal model of chronic aplastic marrow failure was produced by administering busulfan to mice in high dosage for a prolonged period. Mice which survived 60 or more days following cessation of the drug appeared well; in 59% the blood was normal, and in 35% the marrow cellularity was within the normal range; those mice with evident abnormality in the blood and marrow showed only minimal cytopenia in the peripheral blood and moderate decreases in marrow cellularity. Nevertheless, by 240 days 80% of the mice had become ill and died. Sacrifice prior to death showed that aplasia of the marrow and cytopenia (most often pan-cytopenia) were present in the majority of mice. These syndromes of persistent mild marrow hypoplasia and late severe marrow failure which followed busulfan may be suitable experimental models for studying the late effects of myelotoxic agents and the syndrome of chronic aplastic marrow failure in man.

CHRONIC APLASTIC MARROW failure or, as it is more commonly termed, aplastic anemia, is an uncommon but frequently fatal syndrome characterized by pancytopenia, immunologic deficiency,1 marrow hypoplasia or aplasia, and, in the vast majority of cases, the absence of any coexisting hemopoietic disorder capable of causing marrow depression. In approximately one-half of the cases, the syndrome becomes manifest after exposure to a toxic drug or chemical, the classic toxic drug being chloramphenicol. Prophylaxis, early recognition, and treatment of the disease are difficult, owing mainly to the present lack of knowledge of its etiology and pathophysiology. This lack of knowledge is the result of the infrequency and unpredictability of the disease and the ethical impossibility of studying it by reexposing a sufferer to a suspected etiologic agent. Since an animal model of the disease would be of great potential advantage, we have attempted to produce one by repeatedly injecting mice with busulfan. This paper reports our findings.

MATERIALS AND METHODS

Randomly bred virgin female Swiss mice, 12–16 wk of age, were given food and tap water ad libitum. To the water was added 1 g neomycin and 1 ml sodium hypochlorite per liter. Busulfan was dissolved in acetone and the volume made up with sterile water so that a volume of 0.01 ml/g was injected intraperitoneally into each mouse. Two dose schedules were used and were found to give similar results: (1) doses of 20, 20, 20, and 10 mg/kg at two weekly intervals, and (2) weekly injections of 10 mg/kg or less, so that the tibial count was maintained at 5 × 10⁶ cells for 8–9 wk. In a preliminary study the LD₃₀ for a single dose of busulfan was found to be 30 mg/kg.

Mice were not included in the study unless they had survived at least 60 days after the last injection of busulfan. This period of time was arbitrarily chosen to ensure that the animals were no longer affected by any acute toxicity of the drug. In the initial part of the study, mice were randomly sacrificed in groups of four or five at a stage when they appeared well; in the later part...
of the study, as the natural history and high late mortality of the lesion induced by busulfan became apparent, we attempted also to sacrifice mice which looked pale and/or ill. This was done because it was observed that death was frequently preceded by a short period of pallor and/or illness. In some experiments mice were serially bled from the retroorbital sinus and were not sacrificed unless they later appeared unwell.

Blood from the orbital sinus was used only for determining serial hematocrit estimations, and blood obtained by cardiac puncture was used for all other determinations requiring peripheral blood. Suspensions of tibial cells were made by cutting off the ends of the bone, expressing the marrow into saline, and gently drawing the marrow several times through a fine needle. Smears were made from marrow from the femur. Standard techniques were used to examine the peripheral blood and marrow. Leukocyte, platelet, and tibial cell counts were performed visually, reticulocytes were counted as a percentage which was then converted to ml/liter using the corresponding hematocrit estimation, and blood and marrow smears were stained with Wright-Giemsa. Differential leukocyte counts were made on 100 cells, or 50 cells if the total count was very low, and differential marrow cell counts were made on 200–250 cells.

Logarithmic means, standard deviations, and standard errors of the various cell counts were calculated, and comparisons were made using Student's t test.²

RESULTS

The survival of treated and control mice is shown in Fig. 1. These data were obtained from 12 separate experiments in which there were included 190 control mice, 494 mice which commenced treatment with busulfan, 472 mice which survived the course, and 325 which were alive 60 days after cessation of the drug, at which time the study commenced. Survival was followed for a variable time: for at least 120 days following cessation in all 12 experiments and for 240 days in the four most prolonged experiments. It can be seen that following a period

Fig. 1. Survival in control mice and mice treated with busulfan. The left-hand figure is derived from all mice which survived until busulfan was ceased, the right-hand figure from those mice which survived for 60 days after cessation.
of variable duration nearly all of the treated mice eventually died. During this period the mice appeared healthy, but after 200 days they had nearly all developed cataracts. Their weight was less than that of controls, the difference being 6.3 ± 0.8 g (mean ± 1 SE), but this difference did not increase with time, being 6.4 ± 1.2 g for mice sacrificed at less than 100 days and 6.2 ± 1.0 g for mice sacrificed after 100 days. Eighty-two of these mice were sacrificed at intervals ranging from 61 to 175 days after cessation of busulfan. Examination of the blood and marrow of some of these mice showed minor abnormalities (see below), and their hematologic disorder was thus not always truly latent, but, for lack of a better term, these mice will henceforth be referred to as “latent” mice or as mice in the latent stage.

It was possible to detect and sacrifice 53 mice at the stage when they appeared pale and/or ill. Fifteen of the 53 mice proved to have lymphomas or lymphoblasts in the blood or marrow; details of these 15 have been excluded from the present study and will be reported separately. Two of the remaining 38 mice had normal peripheral blood values, and their tibial counts were 8.0 and 9.7 x 10⁶ cells. Although they may have had mild marrow hypoplasia, the normality of the peripheral blood in these animals made it likely that they were ill for some reason other than marrow failure, and, accordingly, their results were also excluded. The remainder of the sick mice will henceforth be referred to as “aplastic” mice, in view of the blood and marrow findings detailed hereunder. The times of sacrifice of this group of aplastic mice ranged from 62 to 313 days after cessation of busulfan.

Figure 2 shows the mean values of the various blood cells in control, latent, and aplastic mice, and Table 1 shows the number of mice examined in each group and the frequency of abnormally low blood counts. On examining the pooled results in the latent group, it is evident that the degree of depression varied, depending on the cell type. The mean number of platelets was not significantly different between the control and latent group; the mean hematocrit, although significantly depressed (p < 0.005), was only slightly so, whereas there

![Fig. 2. Circulating blood cells in control, latent, and aplastic mice. The results are expressed as the mean ± 1 SE, and, except for reticulocytes, the number in each group is as shown in Table 1. Reticulocyte counts were based on 92, 82, and 36 mice for the control, latent, and aplastic groups, respectively.](image-url)
was significant depression ($p < 0.001$) of moderate magnitude in the number of neutrophils, lymphocytes, and monocytes. The number of erythrocytes, neutrophils, and platelets had been simultaneously estimated in 27 mice. All three elements were normal in 16 (59%), one element was depressed to a minimal extent in ten, and two elements were depressed to a minimal extent in one. The 36 aplastic mice showed marked depression ($p < 0.001$) of the mean values of all circulating blood cells when compared with both control and latent mice. In 22 of the 36 aplastic mice erythrocytes, neutrophils, and platelets had been simultaneously estimated. All three elements were depressed in 12 mice, two elements were depressed in five mice, and one element was depressed in five mice. With one exception the mice showing depression of three elements all had tibial counts of $1.7 \times 10^6$ cells or less, whereas those showing depression of two or one elements all had tibial counts of $1.8 \times 10^6$ cells or greater.

Figure 3 shows the results of serial hematocrits performed in individual mice which eventually developed aplasia. It indicates that anemia was not a phenomenon which persisted following busulfan treatment, but that it developed with a varying degree of rapidity as a late event.

![Graph](image-url)
Fig. 4. Total cell counts in the tibias of control, latent, and aplastic mice. The results for the control and latent groups are based on 82 and 71 mice, respectively, and are expressed as the mean ± 1 SD. Individual results for the 36 aplastic mice are shown.

Fig. 5. Histologic appearance of marrow in a control (A) and an aplastic (B) mouse (x 160).
Figure 4 shows the total tibial cell counts in 82 control mice, 71 latent mice, and 36 aplastic mice. The results from the control and latent mice are expressed as the mean ± 1 SD, and individual results from the aplastic mice are shown. The histologic appearance of the marrow from one aplastic mouse is shown in Fig. 5. The mean tibial cell count of mice in the latent stage was 7.0 x 10⁶, which was 58% of and significantly lower (p < 0.001) than the mean control value of 12.1 x 10⁶. Nevertheless, if the lower limit of normal is defined as being 2 SD below the mean for controls, then 35% of the tibial counts of the latent mice were within the normal range. The mean tibial count of mice in the aplastic stage was 1.5 x 10⁶ cells, which was 12% of the mean control value and significantly less (p < 0.001) than the mean for controls and for mice in the latent stage. None of the tibial cell counts of the aplastic mice were within the normal range.

The results of marrow differential counts are shown in Fig. 6. Owing to the paucity of cells present, reliable differential counts could not be performed in many of the aplastic mice, particularly those most aplastic, and hence the results in this group are not shown. However, in view of the severe reduction in total cellularity, the numbers of erythroblasts, neutrophilic granulocytes, and lymphocytes must have been severely reduced. In the latent stage the marrow showed a significant decrease in the number of neutrophilic granulocytes and lymphocytes, but, although there was a slight decrease in the number of erythroblasts, this did not reach statistical significance. In many of the latent mice the decreased number of neutrophilic granulocytes in the marrow appeared to be at least partly due to premature release of the most mature forms.

DISCUSSION

The present study showed that, following chronic administration of busulfan at high dose, those mice which survived the acute toxicity then passed into a latent stage, but eventually most sickened and died. In the minority of cases
death was associated with development of leukemia lymphosarcoma, and in the majority of cases with the development of marrow aplasia and peripheral blood cytopenia. Despite the absent or relatively modest changes in the blood and marrow of latent mice, the subsequent high mortality indicates that at least most of the mice carried a severe defect. Confirmatory evidence for this has been obtained by measuring the numbers of spleen colony-forming units and agar colony-forming cells, which have been found to be reduced to 33% and 17% of control values, respectively. However, it does seem possible that a small proportion of the mice might not have carried a residual defect, or, even if they did, might eventually have either recovered or carried the defect indefinitely without developing marrow failure. During the latent phase the blood showed minimal depression of the hematocrit and moderate depression of the number of neutrophils, lymphocytes, and monocytes, and the appearance of the marrow mirrored that of the blood in that erythropoiesis appeared well preserved compared with granulopoiesis and lymphopoiesis. Differential preservation of erythropoiesis compared with granulopoiesis in a damaged marrow has been noted previously, and conceivably it could be due to some property of the stem cell itself or to a greater sensitivity of the feedback loop controlling erythropoiesis. The depression in the number of marrow lymphocytes and circulating lymphocytes and monocytes is of interest in view of the evidence that monocytopenia and immunologic disturbance commonly occur in aplastic marrow failure in man and in view of the possibility that circulating B lymphocytes may be decreased in the disease.

The hematologic features of mild marrow hypoplasia observed in the latent mice suggest that if a similar phenomenon occurs in man it might be quite difficult to detect. In some instances there might be no decrease in cellularity of blood or marrow; even if present, any decrease in marrow cellularity would not be detectable unless it was marked, owing to the imprecision of the present methods of assessing marrow cellularity in man. Furthermore, any decrease in blood or marrow cellularity might predominantly involve granulopoiesis rather than erythropoiesis and would thus, unless marked, neither produce symptoms nor even lead to a neutrophil count below the lower limit of normal. For these reasons, mild marrow impairment may be not uncommon in man, occurring not only as the result of myelotoxic agents but also on a genetic basis, analogous to the well-recognized hereditary anemias in mice. If, as in the present study, a marrow lesion after toxic agents can persist for a prolonged period of time, one can conceive of a cumulative lesion resulting from summation of the effects of a number of factors acting either simultaneously or sequentially. This type of phenomenon might account for the development of marrow aplasia in patients with infectious hepatitis treated with chloramphenicol, and since minor infections may suppress the marrow, it might account for the observation that when marrow failure occurs following chloramphenicol, it is often after the drug has been prescribed for a relatively trivial infection. Unrecognized preexisting marrow impairment may be important since it may conceivably predispose to frank marrow failure in patients receiving cytotoxic drugs, or may account for some of the cases of idiosyncrasy to drugs such as chloramphenicol.
The present study indicated that frank marrow failure eventually supervened in the majority of mice treated with busulfan. This failure was associated with depletion to less than 1% in the numbers of spleen colony-forming units and agar colony-forming cells. The reasons for the development of failure are at present quite conjectural, but some possibilities are the development in an immunosuppressed host of an infection with a previously latent or exogenously acquired aplasia-producing virus, the development of some nutritional deficiency, the action of an unrecognized toxin, or "senescence" of stem cells leading to failure of proliferation. Understanding the reasons for development of frank marrow failure in busulfan-treated mice may aid in understanding the pathogenesis of chronic aplastic marrow failure in man, and some of the above-mentioned possibilities are amenable to testing.

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