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The Effect of Graded, Single Doses of Busulfan on Murine Erythropoiesis (40744)[1]

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Large, single doses of busulfan (BU) have profound and long-lasting effects on murine hematopoiesis (1–5). Busulfan markedly depletes all classes of putative murine stem cells, including those forming endogenous spleen colonies (1, 5, 8). Busulfan, however, seems to selectively damage the more pluripotent compartments when compared to the more restricted presumed stem cell compartments such as the erythropoietin sensitive cell compartment (3) or the compartment forming granulocytic colonies in semisolid media (3–6). Whole body irradiation (WBI) also has profound effects, but recovery is prompt at sublethal doses (7). Doses of WBI which result in at least a 90% reduction in the hematopoietic stem cell compartment (above 200 rad in most mouse strains) cause a cessation of erythropoiesis and the duration of the no-erythropoiesis interval is directly proportional to the dose (7, 8). Once erythropoiesis resumes following WBI, the slope of its increase is independent of the dose.

This study was designed to explore the dose–response relationship of single doses of busulfan upon erythropoiesis and to compare the qualitative effects of busulfan to those of WBI. The effect on erythropoiesis of doses of busulfan ranging from 10 mg/kg to 100 mg/kg was determined by measuring hematocrit, spleen weight, and radioactive iron uptake into spleen and foreleg at daily intervals in groups of mice following busulfan. Previously reported studies have detailed the effect of 40–60 mg/kg BU upon erythropoiesis, but a dose–response relationship has not been investigated. We were interested in doing so in order to determine if the same qualitative dose–response relationship that was seen after WBI would be seen after BU.

Materials and methods. Female $B_6D_2F_1$ mice, bred in our laboratory from C57Bl♀ and DBA♂ stock, purchased from Jackson Laboratory and 12 to 16 weeks old at the time of study, were housed 10 per cage and given Purine Lab Chow and HCL water (pH 2.4) *ad libitum*. Busulfan (BU) was suspended in warm (37°C) acetone and corn oil, and injected by stomach tube (3, 5). Eighteen hours before they were killed, mice were injected with 0.1 μCi of radioactive iron (^{59}Fe) and immediately before they were killed by cervical dislocation, duplicate microhematocrit tubes were filled by puncturing the lateral orbital sinus. Hematocrit was determined by the micro method in an Adams Autocrit machine calibrated to agree with the Wintrobe method (9). Forelegs and spleens were removed, weighed, and their radioactivity evaluated using a well-type γ scintillation counter. Counts were expressed as a percentage of the total dose of iron which was injected.

Curves of ^{59}Fe uptake into spleen and liver were constructed from mean, daily values in groups of mice given BU 2 to 10 days before. In order to carry out qualitative comparisons with previous studies of the effect of whole body irradiation (WBI) the curves were analyzed in the same way that post-WBI curves were analyzed (6, 7, 10). A baseline, no-erythropoiesis level of iron uptake was established from the nadir of uptake in groups given the highest dose of BU. An abortive wave was defined as a transient increase in ^{59}Fe uptake which then returned to or near the original nadir. The final increase in ^{59}Fe was defined as a fairly

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steady increase toward or above control values. It should be noted that when this final increase in erythropoiesis reaches or exceeds control values, it is maintained for at least a month following single doses of 40 mg/kg of BU (3, 5). Using the first value representing onset of the final increase and values during the increase, but exclusive of values during the subsequent plateau, an exponential function curve was calculated by the method of least squares. The slope of the curve of increase was compared for similarities or differences after various doses. The time of "onset" of erythropoiesis was calculated by projecting the curve of increase to its intersection with the basal, no-erythropoiesis level. The time of the intersection of the curves was rounded off to the nearest 0.5 days. As can be noted by comparing the curve of uptake after 40 mg/kg in Fig. 1 and the calculated time of onset for those groups in Table 2, this statistical treatment not unexpectedly "idealizes" the curves to a modest degree. Means, standard errors, differences between groups using χ^2 or Student's t test and correlation coefficients were calculated by standard statistical means. All reported ^{59}Fe iron values were rounded to the nearest first decimal.

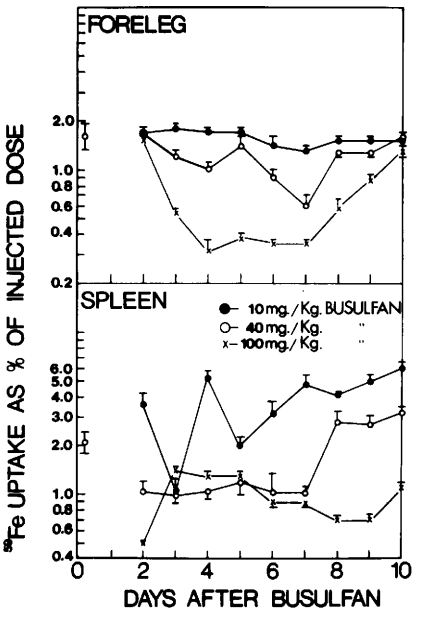

FIG. 1. *The effect of 10, 40, and 100 mg/kg of busulfan on erythropoiesis as reflected in uptake of ^{59}Fe by marrow (foreleg) and spleen.* Control values for uninjected mice are shown on Day 0. Mean and standard error for 10 mice per group are shown by points and vertical bars on Days 2–10 postbusulfan.

Results. Six groups, 90 mice per group, were given 10, 20, 40, 60, 80, or 100 mg of busulfan (BU) per kilogram, and 10 from each group were killed on Days 2 through 10. Mild to moderate anemia was produced in all mice (Table I). The severity of the anemia was directly proportional to the dose of busulfan, and the nadir in hematocrit occurred at 6–9 days after busulfan. In all groups, except the one given 100 mg of busulfan, the hematocrit had risen significantly above its nadir by 10 days.

Changing ^{59}Fe uptake curves for foreleg and spleen following 10-, 40-, and 100-mg doses of BU are shown in the figure and what we consider to be key events (as analyzed for postirradiation studies, see methods) in curves for all doses are shown in Table II. These events (Fig. 1) are the initial nadir, an abortive rise, a second decrease and a final rise as well as the calculated day of resumption of erythropoiesis (see Materials and Methods.). A confirming study was done using 10 and 100 mg/kg of BU in which very similar results were obtained to those shown. When groups of mice given 40–60 mg/kg of BU were followed for longer periods of time than those shown in the table or figures, all values for erythropoiesis eventually became normal and remained so for at least a month (3, 5).

In general, the changes in iron uptake which were observed in the spleen were exaggerated as compared to those seen in the marrow although the overall pattern tended to be the same in both organs.

In the marrow, neither 10- or 20-mg doses produced appreciable effects although a transient but significant ($P < 0.05$) reduction in ^{59}Fe uptake was noted after 20 mg. An initial nadir occurred at a median of 4 days and its severity was directly proportional to the dose of BU ($r = 0.99$, $P < 0.001$). There was a suggestion of an abortive rise, peaking 1 day after the initial nadir in all groups receiving 40 mg or more of BU but the rise differed significantly from the initial and subsequent nadir only in the

TABLE I. HEMATOCRIT AND SPLEEN WEIGHT VALUES FOLLOWING DIFFERENT DOSES OF BUSULFAN

Dose of busulfan (mg/kg)	Hematocrit (%)[a]		Spleen weight (mg)[a]	
	Lowest value (day)	Value at 10 days	Lowest value (day)	Value at 10 days
10	44 ± 0.8 (8)	46 ± 0.8	54 ± 0.8 (3)	79 ± 3.5
20	41 ± 1.0 (6)	46 ± 0.4	55 ± 1.5 (2)	71 ± 4.2
40	34 ± 0.9 (7)	42 ± 1.1	49 ± 2.2 (7)	71 ± 3.1
60	35 ± 1.0 (8)	40 ± 1.2	47 ± 2.1 (7)	66 ± 2.5
80	30 ± 0.5 (8)	36 ± 1.2	45 ± 2.6 (6)	55 ± 2.0
100	29 ± 1.1 (9)	30 ± 1.3	39 ± 3.4 (6)	48 ± 1.1

[a] Mean control hematocrit of untreated mice is 49% and mean spleen weight is 65 mg.

TABLE II. ^{59}Fe UPTAKE AND TIME OF FIRST NADIR, ABORTIVE RISE, SECOND NADIR, AND FINAL 10-DAY VALUES AFTER GRADED DOSES OF BUSULFAN

Dose of busulfan (mg/kg)	First nadir (day) $\bar{X}$	^{59}Fe uptake as percentage of injected dose: Abortive rise (day) $\bar{X}$	Second nadir (day) $\bar{X}$	Final peak (10 days) $\bar{X}$	Day of onset of final rise in Fe uptake
Foreleg[b]					
10	NE[a]	NE	NE	1.5	NE
20	(6) 1.2	NE	NE	1.7	NE
40	(4) 1.0	(5) 1.4	(7) 0.6	1.6	5.5
60	(3) 0.8	(4) 0.9	(7) 0.6	1.6	6.0
80	(4) 0.4	(5) 0.5	(7) 0.4	1.5	6.5
100	(4) 0.3	(5) 0.4	(7) 0.3	1.3	7.0
Spleen[b]					
10	(3) 1.0	(4) 5.4	(5) 2.0	6.1	3.0?
20	(4) 1.3	(5) 1.7	(6) 1.6	3.7	5.5
40	(3) 1.0	(6) 1.2	(7) 1.0	3.2	6.0
60	(2) 0.7	(5) 1.4	(6) 0.9	2.6	6.5
80	(2) 0.8	(4) 1.3	(8) 0.6	2.2	7.5
100	(2) 0.5	(3) 1.4	(8) 0.7	1.1	9.0

[a] This value was not evident (NE) from the curves.
[b] Value for iron uptake in controls was 1.6% for foreleg, 2.1% for spleen.

group receiving 40 mg/kg ($P < 0.01$). Following the abortive rise, the second nadir appeared at a median time of 7 days and was as low or lower a value as during the first nadir in all groups. The severity of the second nadir also tended to be in direct proportion to dose. By 10 days, control values had been achieved by all groups except those which received 100 mg and the calculated time of onset for resumption of erythropoiesis was directly proportional to the dose of BU ($r = 1.0$, $P < 0.001$); there being an average delay of 0.5 days per increment of 20 mg BU between doses ranging from 40 to 100 mg.

In the spleen, there was a more evident effect of 10 and 20 mg BU than in marrow (Fig. 1, Table I). The severity of the first nadir tended to be directly proportional to dose of BU but this relationship was more erratic ($r = 0.86$, $P < 0.001$) than in marrow and occurred earlier (median 2.5 vs 4 days). The abortive rise was more convincing in spleen than in marrow in that there was a significant increase ($P < 0.05$) as compared to the first or second nadir figures after 10-, 60-, 80-, and 100-mg BU doses and it occurred approximately at the same time as in marrow (median peak value at 4.5 vs 4.0 days). The second nadir tended to be less severe in spleen as compared to marrow in that only 2/6 were equal to or as low as the first as compared to all being such in marrow. The calculated time of onset of resumption of final erythropoiesis was directly proportional to dose ($r = 0.97$, $P < 0.001$) but was later in spleen as compared to marrow and the delay/20-mg dose was greater ($\sim$ 1 day extra delay/20-mg dose vs $\sim$ 0.5 day). However, once erythropoiesis resumed, the slope of the increase in iron uptake was steeper in spleen than in marrow and except for the group receiving 100 mg, all exceeded mean control values by 10 days. The height of the 10-day ^{59}Fe value in spleen was inversely proportional to BU dose.

The slope of final increase was not significantly different between groups given different doses of BU in marrow nor was it different between groups in spleen.

Spleen weight was also significantly affected, and the degree of decrease tended to be directly proportional to the dose of BU (Table I). Most iron taken up by the spleen of a mouse represents erythropoiesis in the spleen, but even in the absence of any erythropoiesis the minimal residual iron uptake is still directly proportional to spleen weight. To be certain that the differences in iron uptake between the groups reflected erythropoiesis rather than merely differences in spleen weight, ^{59}Fe/mg of spleen was calculated. These curves (not shown) were very similar those for total iron uptake per spleen (Fig. 1B).

Mouse weight (data not shown) tended to decline in direct proportion to the dose of busulfan. Spleen colonies were also counted (data not shown) and as noted previously these are often ill defined and difficult to count following busulfan (5, 6) but, in general, changes in their number tended to parallel iron uptake.

Discussion. The primary purpose in undertaking the present study was to determine if the pattern of interruption and resumption of erythropoiesis following damage by graded doses of busulfan (BU) was similar to that seen after graded doses of whole body irradiation (WBI), taking into account the known differences in the effect of these treatments upon hematopoietic stem cells (1–8, 10). The time of onset of erythropoiesis following its interruption by WBI was directly proportional to the dose of WBI (7, 8). Further, where mice were lethally irradiated and given marrow the time of onset of erythropoiesis was inversely proportional to the marrow cell dose (10).

The pattern of erythropoiesis following a single dose of BU is quite similar to that reported by Reissmann and co-workers (3). The studies of Morley (11) indicate a more profound and long-term effect of BU but represent the effect of repetitive doses of BU.

The degree of depression in erythropoiesis was directly proportional to the dose of BU as measured by degree of anemia and depression in uptake of ^{59}Fe by spleen and marrow. Erythropoiesis may not have been completely abolished in any group as the minimum value was never as low as values seen after large doses of ir-

radiation. Following doses of WBI exceeding 200 R, ^{59}Fe per spleen decreases to less than 0.3% of the injected dose (7, 8), while the lowest post-BU value was 0.5%. However, since spleen weight was not reduced to as great a degree after BU as compared to WBI, this may not reflect residual erythropoiesis. There was a suggestion of abortive rises in erythropoiesis as measured by iron uptake into spleen and marrow. Abortive rises in erythropoiesis as well as various other measures of hematopoiesis following BU have been reported previously (1–5). No abortive rise in erythropoiesis follows WBI unless the mouse is stimulated by bleeding or injecting erythropoietin within a few hours after WBI (8, 10). The reason why these abortive rises occur is unclear (3–5, 8, 10) although it has been suggested that the abortive rise in erythropoiesis is the result of stimulation of a specific type of stem cell (12).

BU has been shown to have a much greater effect on transplantable cells forming spleen colonies (CFU) than it does on cells forming colonies of granulocytes and macrophages in soft agar (CFU-C) (1–3, 5). It is believed that the CFU-C is the progeny of the pluripotent CFU. Failure to completely ablate erythropoiesis with busulfan may possibly reflect a lesser effect of the drug on the erythropoietin sensitive cell than on its presumed parent, the CFU, as has been suggested by other studies (3).

The present data are qualitatively similar to those following WBI if it is assumed that the secondary increase reflects differentiation from the pluripotent stem cell compartment and the changes occuring before the secondary rise are taken to reflect the lesser damage of committed precursors and ignored. If the slopes of the curves for the secondary rise are projected back to a theoretic no-erythropoiesis level, the time of onset of erythropoiesis is directly proportional to the dose of BU, as it is following WBI (7, 8) or after giving graded doses of marrow cells to lethally irradiated mice (10). Regrowth of endogenous CFU (E-CFU) following 40–60 mg of busulfan becomes most readily apparent at 5–6 days and of exogenous CFU (CFU-S) at 12–14 days although there is a modest increase in the latter between 1 and 10 days (5). Resumption of erythropoiesis following these doses was at 5 to 6 days in marrow and 6 to 7 days in spleen; compatible with stem cell regrowth preceeding differentiation.

Splenic erythropoiesis generally reflected the same pattern of changes as was seen in marrow although there were significant differences in degree and in timing between the two organs. This similarity generally has been observed in other studies (5, 6, 8, 13) but caution is indicated in generalizing about hematopoietic changes, based on changes in the murine spleen. In response to a very wide variety of stimuli, splenic erythropoiesis increases while marrow erythropoiesis decreases (13). However, except for studies in which the marrow has been ablated (14), marrow erythropoiesis still appears to exceed splenic hematopoiesis. Normally, only 2–3% of total erythropoiesis takes place in the spleen and even with very strong, but non-marrow ablative stimuli, it has not been reported to exceed total marrow erythropoiesis. Thus, although splenic erythropoiesis clearly is important physiologically (13), unless changes in it are reflected in marrow to some degree, as they were in the present study, they may be difficult to interpret with respect to total erythropoiesis.

Our previous studies of the relationship of the time of onset of erythropoiesis to the size of the pluripotent hematopoietic stem cell compartment (HSC) in either sublethally irradiated (7, 8) or lethally irradiated mice transplanted with marrow (10) have led to the following hypothesis; an hypothesis also formulated by other investigators based on somewhat related data in mice (15). If HSC are reduced to less than 10% of normal, some form of recognition of compartment depletion develops within a few hours and under this circumstance, little or no differentiation can occur from HSC until the compartment regrows to above 10% of normal by the process of self-replication. Differentiation is equivalent to death for a population of stem cells and the period of no-erythropoiesis is one in which the number of stem cells, as measured by number of endogenous spleen colonies, or

transplanted spleen colonies, is increasing exponentially. Consequently, this period of stem cell self-replication without differentiation represents a means by which the depleted stem cell pool is protected from potential exhaustion. These data on the effects of BU on erythropoiesis are compatible with, but in no sense prove, this hypothesis.

Summary. The effect of busulfan on murine erythropoiesis was studied following doses ranging from 10 to 100 mg/kg. The degree of depression of erythropoiesis was directly proportional to the dose of busulfan, as measured by hematocrit changes or by uptake of radioactive iron by spleen and marrow. The pattern of postbusulfan erythropoiesis was different from that seen after sublethal doses of whole body irradiation in some respects in that an abortive wave of erythropoiesis occurred in both marrow and spleen prior to onset of a more substantially sustained erythropoietic recovery. The pattern was like that following radiation in that the degree of reduction of erythropoiesis was directly related to dose as was the interval of time prior to onset of final recovery. In the spleen, this interval was 1 day per 20 mg/kg and in the marrow 0.5 days per 20 mg/kg.

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