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Reduction of Busulfan-Induced Granulocytopoietic Toxicity in Mice by Postbusulfan Injection of Endotoxin (40885)[1]

SALLIE S. BOGGS[2], PAUL A. CHERVENICK, AND DANE R. BOGGS

Departments of Medicine and Radiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Abstract. Busulfan produces profound and prolonged hematopoietic depression in mice. However, the decline in pluripotent cell compartments such as those forming spleen colonies (CFU-S) is much more precipitate than the decline in their progeny such as cells forming colonies in methylcellulose (CFU-C) or in granulocytes. Endotoxin was given immediately following a single 60 mg/kg dose of busulfan in order to determine its influence upon the subsequent decline and recovery of CFU-C, total nucleated cells, and marrow granulocytes/humerus. There was no effect on the rate of decline for 3 days following busulfan. However, the decline slowed in endotoxin treated mice on the 4th day and all three values were significantly higher than in controls on Day 5, the nadir for controls. Controls had an abortive recovery evident by Day 7. At this time endotoxin treated mice had higher values than in controls for total nucleated cells and granulocytes, but not for CFU-C. By Day 9, no further effect of endotoxin could be demonstrated. This effect is similar to that of androgenic steroids on the granulocytic system of busulfan-treated mice. The simplest explanation for the effect is to suggest that CFU-C are capable of self-replication and that endotoxin stimulates this self-replication.

A single dose of busulfan (Myleran) causes a severe and prolonged depression of all hematopoietic cell lines (1, 2). Cells forming spleen colonies, either by transplantation (CFU-S) or studied endogenously (E-CFU), are reduced to very low levels almost immediately following busulfan. Cells forming colonies of granulocytes and macrophages in semi-solid media (CFU-C) decline more slowly than CFU-S and E-CFU. Erythropoiesis and granulocytopoiesis, as measured by production of mature cells, show a gradual decline and make an abortive recovery before significant recovery of the pluripotent spleen colony forming stem cells is observed. The abortive wave of increased erythropoiesis can be accentuated by injection of erythropoietin (3). These results are in accord with other evidence suggesting a degree of self-replicative ability for "committed" erythropoietin sensitive precursors (4) and a role for erythropoietin in proliferative as well as differentiative stimulatory regulation (5). Similarly, when androgenic steroids were given following busulfan, the abortive wave of CFU-C recovery was accentuated, suggesting self-replicative potential for this cell (6).

Studies in our laboratory have led to the hypothesis that when the stem cell compartment is diminished below a certain threshold, differentiation into committed cell compartments ceases until the pluripotent compartment has had time to repopulate to a threshold level estimated at about 10% of normal (7). For a short period (less than 1 day) after depletion of stem cells by irradiation, bleeding or injection of endotoxin will elicit an abortive wave of erythropoiesis or granulocytopoiesis, respectively. The prolonged depression of CFU-S below the estimated 10% "threshold" level after busulfan treatment afforded an opportunity to study the various precursor compartments and their regulation.

The present studies were undertaken to determine if postbusulfan injection of en-

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dotoxin would modify granulocytopoietic toxicity in a manner analogous to that seen for erythropoiesis after injection of erythropoietin. Mice were given busulfan, followed by endotoxin, and total nucleated cells per humerus, peroxidase positive cells per humerus, and CFU-C were monitored during the succeeding 2 weeks. For a detailed consideration of the effect of busulfan alone on these measures of hematopoiesis and also on transplanted and endogenous spleen colonies, hematocrit, spleen weight, and iron uptake per spleen and humerus, please see Boggs *et al.* (1).

Materials and methods. Female $B_6D_2F_1$ mice, bred in our laboratory from C57 BL/6 females and DBA/2 males purchased from Jackson Laboratories, were 12–21 weeks of age, housed 5–10/cage, and given Purina Lab Chow and HCl water (pH 2.4) *ad libitum*. Busulfan was dissolved in warm (37°C) acetone, diluted in warm corn oil (1:5), and injected by stomach tube in a dose of 60 mg/kg body wt in 0.03 ml/g. Endotoxin, 25 μg, Difco Laboratories, was injected i.p. within 1 hr after busulfan. The total number of nucleated cells in a humerus was measured by quantitative washout and counting an aliquot of the washout electronically (8). Another aliquot was dispersed on millipore filters and stained for the peroxidase reaction, and 300–500 cells were enumerated as peroxidase positive or negative.

For determination of *in vitro* growth of granulocytic and macrophage colonies, femoral marrow aliquots were studied by an adaptation of the method described by Pluznik and Sachs (9) and Bradley and Metcalf (10) as modified by Worton, McCulloch, and Till (11). Briefly, cells were washed from the femurs and 75,000 nucleated marrow cells were suspended in 1.2% methylcellulose containing tissue culture medium (CMRL 1066) and 15% horse serum. Colony stimulating factor source was conditioned media from mouse L-cells. One milliliter of the combined mixture was plated into 10 × 35-mm petri dishes and incubated at 37°C in 7.5% CO_2. After 7 days of growth, the number of colonies (containing greater than 50 cells) was counted with the aid of an inverted microscope as described previously (12).

The number of exogenous colony forming units (CFU-S) obtainable from the marrow of one humerus was evaluated by injecting 0.5-ml aliquots of cells equivalent to 1/100th or 1/10 humorus i.v. into recipients which had been given 900 rads γ radiation less than 3 hr earlier. Recipient mice were killed 8 days after irradiation and spleens were removed and placed in Bouin's fixative. Counts of any colonies present on the spleens were made by three individuals and when different counts were recorded an average was taken.

In all experiments, control and experimental groups were studied on the same day.

Results. Three studies were done in which mice were or were not given endotoxin immediately after a dose of 60 mg/kg of busulfan. Other controls given nothing or endotoxin but no busulfan also were studied each day, but as there was minimal variation from day to day, these results are not shown except as a mean control value for Day 0. Groups were killed at 1 to 3-day intervals for 14 days after busulfan and total nucleated cells (TNC) per humerus were measured. As the results of all studies were similar, the data were pooled. It must be emphasized that the differences referred to as significantly different for the pooled data were observed to be significantly different in each of the three replicate studies.

Total nucleated cells per humerus fell steadily, reaching a nadir of 15% of untreated controls by Day 5 after busulfan, then increased for 2 days but plateaued at values less than normal by 14 days (Fig. 1). Following endotoxin, the initial rate of fall was as rapid for the first 3 days as in busulfan-treated controls. However, the rate of decrease slowed between Days 3 and 4 and the increase began a day earlier than in controls. Values for endotoxin-treated mice were significantly higher than in controls on Day 5 ($P < 0.05$). From Days 7 to 14 there was no statistically significant difference between the groups.

The effect of endotoxin on TNC was primarily due to changes in granulocytes.

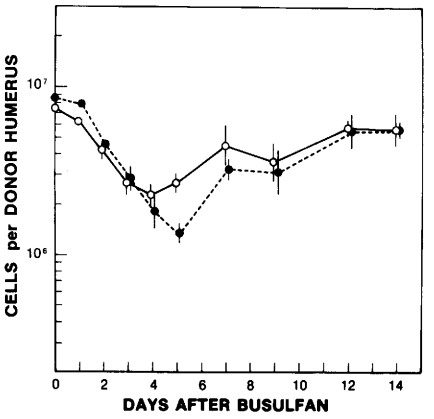

FIG. 1. The effect of 25 μg *S. typhosa* endotoxin given immediately after a dose of 60 mg/kg busulfan on the total number of nucleated cells per humerus. Each point represents the mean of at least 10 mice and standard errors are shown. Open circles and solid line represent mice given endotoxin after busulfan. Closed circles and dashed line represent mice given busulfan only.

Following busulfan, peroxidase positive cells followed the same curve of decrease and increase in TNC (Fig. 2). Endotoxin-treated groups fell as rapidly as controls for the first 3 days but began to increase a day earlier and reached a higher peak on Day 7, being significantly higher than control values on Days 4, 5, and 7. Endotoxin had no significant effect on values at 9, 12, and 14 days.

Values for CFU-C reached a nadir on Day 9 following busulfan with an abortive rise on Day 2 and a second abortive rise on Day 7 (Fig. 3). A slow rise occurred between Days 9 and 14. The endotoxin treated group did not decrease to as great a degree as controls on Day 1 and did not have an abortive rise on Day 2. From Days 3 to 5 the rate of decrease slowed in endotoxin treated mice so that the Day 5 value was significantly higher than in controls, but thereafter there was no difference between the groups.

CFU-S also were measured, but have been published in another paper describing the effects of busulfan on many hematopoietic systems (1). By 1 day after 60 mg/kg busulfan the CFU-S had dropped from 2100 ± 80 per humerus in nontreated controls (183 mice) to 11 ± 4 in busulfan-treated mice (0.5% of normal). The numbers of CFU-S on Days 2, 4, and 6 were between 23 and 43 and Days 8, 10, and 12 were between 58 and 69 per humerus. Even

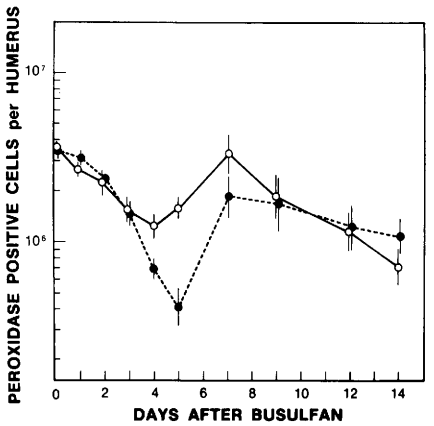

FIG. 2. The effect of endotoxin given after busulfan on peroxidase positive cells (granulocytes) per humerus. Each point represents the mean of at least 10 mice and standard errors are shown. Open circles and solid line represent mice given endotoxin after busulfan. Closed circles and dashed line represent mice given busulfan only.

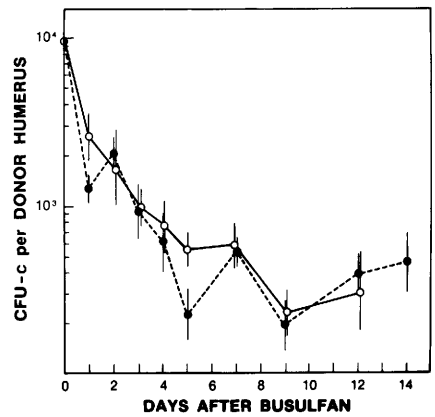

FIG. 3. The effect of endotoxin given after busulfan on cells forming colonies in methylcellulose (CFU-C). Each point represents the mean of at least 10 mice and stand errors are shown. Open circles and solid line represent mice given endotoxin after busulfan. Closed circles and dashed line represent mice given busulfan only.

on Day 14 there were only 103 ± 39 CFU-S per humerus. CFU-S were not measured in busulfan-treated mice given endotoxin.

Discussion. These data indicate that endotoxin, given immediately after busulfan, significantly modified busulfan-induced granulocytopoietic toxicity. This effect was primarily a modification of the severity of decline observed at 5 days after busulfan. Two weeks following busulfan, recovery was incomplete and recovery at that point was not influenced by endotoxin.

Reissman and Samorapoompichit (3) concluded that the effect of postbusulfan erythropoietin on the erythropoietin-sensitive-cell compartment represented a stimulation of self-replication in that compartment. They felt it unlikely that enough CFU-S remained to produce the effect as mediated by a CFU-S to erythropoietin-sensitive-cell differentiation process. Similarly, they concluded that the effect of androgenic steroids on CFU-C and granulocytopoiesis following busulfan was indicative of self replication in the CFU-C compartment (6).

In many studies the effects of endotoxin on granulocytopoiesis are qualitatively similar to the effects of erythropoietin (or bleeding) on erythropoiesis (7). This is true of the present study in that endotoxin modified the initial severity of granulocyte depletion without an effect on final recovery, similar to erythropoietin's effect on postbusulfan erythropoiesis (3).

Firm proof that CFU-C are capable of self-replication is lacking. However, the size of the colonies that are produced from single cells and the slow decline in such cells after busulfan in the face of almost absent CFU-S (1, 6) suggests self-replication can occur. The simplest explanation for the post-busulfan effect of endotoxin would be to suggest it stimulates self-replication in the CFU-C compartment. If self-replication was stimulated, compartment size would decline more slowly, total feedout would be increased into peroxidase positive cells, and the latter would also decline more slowly than in mice not given endotoxin. Alternatively, the effect could be due to endotoxin stimulating increased feed out from the CFU-S, E-CFU compartment(s) into the CFU-C compartment. This would result, presumably, in a further decrease in the size of the former compartments. We did not attempt to assess such a possible change for technical reasons; E-CFU are undetectable and CFU-S are reduced to 0.5% or less than normal 1 day after busulfan (1). We think the severe reduction in these compartment sizes makes this an unlikely explanation. Further, if increased CFU-S, E-CFU differentiation occurred at the expense of their self-replication, the recovery phase should be delayed, and it was not.

The effect of endotoxin could be directly on the cell or mediated by endotoxin's stimulation of release or production of hormonal stimulating factors. All putative humoral regulating factors of granulocytopoiesis are stimulated by endotoxin, colony stimulating factor (13), diffusable granulocytic stimulating substance (14), and neutrophil releasing factor (15).

We assume that the time of final increase in granulocytopoiesis from its postbusulfan nadir reflects the rate of regrowth of pluripotent stem cells (1), a process apparently unaffected by endotoxin given after busulfan (7).

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