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# Response of Erythroid Day 3 Burst-Forming Units to Endotoxin and Erythropoietin (40664)

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In plasma clot and methyl cellulose cultures containing erythropoietin (Ep), morphologically unidentifiable erythroid progenitor cells proliferate and differentiate *in vitro* forming clusters of erythroblasts (1, 2). By size, configuration and time of appearance of *in vitro* clusters, and by physical and biological properties of the progenitor cells, murine erythroid progenitors have been classified into three stages: colony-forming units (CFU-E), Day 3 burst-forming units (Day 3 BFU-E), and Day 8 burst-forming units (Day 8 BFU-E) (1, 3, 4). The CFU-E give rise to small colonies of eight or more erythroblasts at 2 days of culture with small concentrations of Ep and have a modal sedimentation velocity at unit gravity of 7.0 mm/hr, whereas the Day 8 BFU-E give rise to clusters of erythroblast colonies at 8 days of culture, require larger amounts of Ep and have a modal sedimentation velocity at unit gravity of 3.8 mm/hr (1, 5). Day 3 BFU-E are intermediate in these characteristics (5). These three stages are considered to be part of a continual, multistaged process of erythroid proliferation and differentiation which begins with the Day 8 BFU-E and proceeds through the Day 3 BFU-E stage and subsequently through the CFU-E stage yielding erythroblasts (6–8).

When normal mice were injected with endotoxin, erythropoiesis increased in the spleen and decreased in the marrow (9, 10). However, in plethoric mice, whose Ep levels are very low, endotoxin increased the number of splenic CFU-E, but not the number of erythroblasts (11). The fact that increased numbers of erythroblasts accompanied increased numbers of CFU-E in normal mice given endotoxin, but not in plethoric mice, suggested that the presence of Ep enhanced differentiation of the splenic CFU-E into erythroblasts, but that endotoxin did not require the presence of Ep to increase the number of splenic CFU-E. Because the increase in splenic CFU-E induced by endotoxin was delayed and occurred 24 hr following a single injection, with a maximal increase 72 to 96 hr after injection, Reissmann *et al.* (11) postulated that the increase in splenic CFU-E seen after endotoxin administration was the result of a primary effect of endotoxin on an earlier precursor cell. No further investigation has been reported, however, on the effect of endotoxin on the earlier splenic Day 3 BFU-E. Endotoxin has been identified as a contaminant in Ep preparations (12–14) used by almost all investigators in the study of erythropoiesis, and has been shown to inhibit stimulation of erythropoiesis *in vivo* by these Ep preparations (14). The present study was undertaken to determine the effect of endotoxin on the number of marrow and spleen Day 3 BFU-E with and without the presence of Ep. Using both morphological and biochemical studies of cells from hypertransfused mice, we have found that endotoxin markedly increases the number of splenic Day 3 BFU-E and decreases the number of these cells in the marrow. Endotoxin had little effect on Day 3 BFU-E differentiation. In contrast to the effect of endotoxin, the major effect of Ep was to increase the number of more differentiated erythroid precursor cells, including the CFU-E, in the marrow and spleen. Spleens of plethoric mice given both endotoxin and Ep had increased numbers of the more differentiated erythroid progenitors as observed in mice given Ep alone and had decreased numbers of the earlier erythroid progenitors compared to mice given endotoxin alone.

*Materials and methods.* Male BALB/c mice from 9 to 12 weeks of age and approximately 20 g were made plethoirc by i.p. injection of 0.5 ml of packed BALB/c erythrocytes on Day 1. On Day 3, mice with microhematocrits ranging from 62 to 65% (normal 48–50%) were selected for treatment on Days 4–7.



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Groups of five or six mice were treated as follows: (i) control—no treatment; (ii) endotoxin treated—daily i.p. injections of 10 μg endotoxin (lipopolysaccharide B *S. enteriditis*, Difco) diluted with Hanks' balanced salt solution (HBSS); (iii) Ep treated—twice daily i.p. injections of 0.5 units sheep Ep (Connaught, Step 3) which had been diluted in HBSS and from which endotoxin had been removed as described by Graber *et al.* (15) with limulus amebocyte lysate (Worthington); (iv) endotoxin + Ep treated—daily injections of 10 μg of endotoxin plus twice daily injections of 0.5 units of limulus-absorbed Ep. On Day 8 hind limb marrow cells were obtained by flushing femoral and tibial marrow cavities with HBSS and then gently passing the cell suspensions through a 27-gauge needle. Spleen cells were obtained by straining sliced spleens through nylon mesh bags from human platelet administration sets. After determining nucleated cell counts with a hemocytometer, the cells were suspended in tissue culture media containing 1.0 unit/ml of limulus-absorbed Ep and incubated at 37° in air plus 5% $CO_2$.

To quantitate colonies and bursts, cells were cultured in plasma clots consisting of α-medium with 30% heat-inactivated (56° for 60 min) fetal calf serum (Grand Island Biological Co.), 1% deionized bovine serum albumin diluted with phosphate-buffered saline (PBS), $10^{-4}$ *M* β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.85% beef embryo extract (GIBCO), and 10% citrated bovine plasma (GIBCO) as described by McLeod *et al.* (16). For determination of CFU-E, suspensions of $5 \times 10^5$ nucleated cells/ml were cultured in quintuplicate 0.1-ml aliquots in microtiter wells (Linbro). At 36 hr of incubation, the clots were fixed with 5% glutaraldehyde, stained with benzidine and hematoxylin, and colonies of eight or more benzidine-positive cells were counted, For determination of Day 3 BFU-E, suspensions of $4 \times 10^5$ nucleated cells/ml were incubated in triplicate 0.5-ml aliquots in 16-mm-diameter wells (Costar). The clots were fixed and stained at 66 hr of incubation and scored according to the criteria of Gregory (4).

For determination of $^{59}Fe$ incorporation into heme, cells were incubated in α-medium with 0.8% methyl cellulose, 30% fetal calf serum, 1% bovine serum albumin, $10^{-4}$ *M* β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin using a slight modification of the method of Iscove and Sieber (2). Cell suspensions of $2 \times 10^6$ nucleated cells/ml were incubated in 0.1-ml aliquots in microtiter wells. On each of 7 or 8 consecutive days, five wells were pulsed for 24 hr with 12 μl of a 10 μCi/ml solution of $^{59}FeCl_3$ in iron-poor human plasma using a slight modification of the method of Eliason *et al.* (17). At the end of each 24-hr pulse, the cells were removed from the wells, washed twice with 1 ml of PBS, and lysed with 0.9 ml of 33% Drabkin's solution. After acidification with 0.1 ml of 1 *N* HCl, heme was extracted into an equal volume of cyclohexanone and $^{59}Fe$ uptake was determined as cpm/culture in an automatic gamma well-type scintillation counter. Greater than 95% of cyclohexanone-extractable $^{59}Fe$ was found with mouse hemoglobin that was chromatographed on CM-cellulose columns. Statistical significance was determined by Student's *t* test.

*Results.* Marrow and spleen cells from control mice and mice treated with endotoxin, Ep, or endotoxin + Ep were cultured simultaneously for determination of CFU-E, Day 3 BFU-E, and $^{59}Fe$ incorporation into heme. The number of erythroid colonies in each group was proportional to Day 1, 0- to 24-hr, $^{59}Fe$ incorporation, and the number of Day 3 bursts was proportional to Day 3, 48- to 72-hr, $^{59}Fe$ incorporation (Table I). Because we wished to observe the cumulative kinetic effect of Ep *in vitro* on the erythroid precursor cell compartment, further experiments were performed with daily measurements of $^{59}Fe$ uptake. By using 1.0 unit/ml of limulus-lysate-absorbed Ep in the medium, marrow and spleen cell cultures from treated and untreated mice had their highest $^{59}Fe$ incorporation within the first 5 days of incubation (Figs. 1, 2). As several experiments showed low $^{59}Fe$ incorporation on Days 6–8, subsequent experiments were terminated after 7 days of incubation.

Endotoxin-treated mice had no significant increase over nontreated mice in number of splenic nucleated cells, but they had a very marked increase in Day 3 BFU-E and Days 3–5 $^{59}Fe$ incorporation (Table I, Fig. 1). A

TABLE I. COMPARISON OF CFU-E AND DAY 1 $^{59}$Fe INCORPORATION, PLUS DAY 3 BFU-E AND DAY 3 $^{59}$Fe INCORPORATION, OF SPLEEN OR MARROW CELLS FROM HYPERTRANSFUSED MICE TREATED FOR 4 DAYS WITH ENDOTOXIN, ERYTHROPOIETIN (Ep), OR ENDOTOXIN + Ep

| Organ Treatment | CFU-E[a] ($\times 10^{-3}$) | Day 1 Heme $^{59}$Fe[a] (cpm $\times 10^{-3}$) | Day 3 BFU-E[a] ($\times 10^{-3}$) | Day 3 Heme $^{59}$Fe[a] (cpm $\times 10^{-3}$) |
|---|---|---|---|---|
| Spleen | | | | |
| None | 0.0 ± 0.0 | 9.0 ± 1.2 | 11.9 ± 1.6 | 33.8 ± 4.0 |
| Endotoxin[b] | 19.5 ± 5.4 | 66.6 ± 4.6 | 93.7 ± 5.2 | 620.8 ± 12.4 |
| Ep[c] | 62.7 ± 5.5 | 493.9 ± 19.2 | 39.2 ± 4.7 | 158.7 ± 9.8 |
| Endotoxin + Ep[d] | 189.1 ± 25.9 | 1054.1 ± 61.5 | 243.5 ± 24.6 | 1305.3 ± 100.4 |
| Marrow | | | | |
| None | 3.6 ± 0.4 | 5.1 ± 0.4 | 18.9 ± 1.2 | 60.5 ± 3.1 |
| Endotoxin[b] | 0.2 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.0 | 8.6 ± 0.5 |
| Ep[c] | 26.4 ± 2.9 | 45.1 ± 1.5 | 20.7 ± 1.6 | 59.7 ± 2.3 |
| Endotoxin + Ep[d] | 2.4 ± 0.3 | 5.0 ± 0.1 | 5.8 ± 0.5 | 32.3 ± 1.7 |

[a] Per spleen or hind limb ±SEM.
[b] 10 μg/day i.p.
[c] 0.5 unit × 2/day i.p.
[d] 10 μg/day + 0.5 units × 2/day i.p.

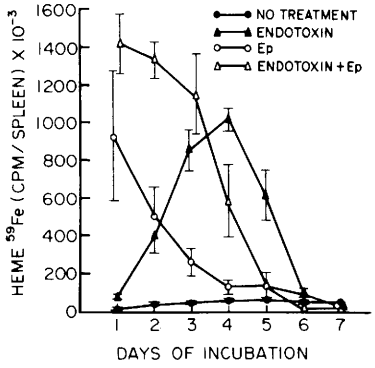


FIG. 1. Daily $^{59}$Fe incorporation into heme of cultured spleen cells from hypertransfused mice. After 4 days of treatment, nucleated cell counts × $10^{-6}$/spleen were: no treatment, 151.7 ± 7.5; endotoxin, 163.5 ± 2.8; Ep, 192.0 ± 17.0; endotoxin + Ep, 193.0 ± 5.1. Data represent mean ±SEM for six experiments except Ep and endotoxin + Ep groups which represent three experiments.

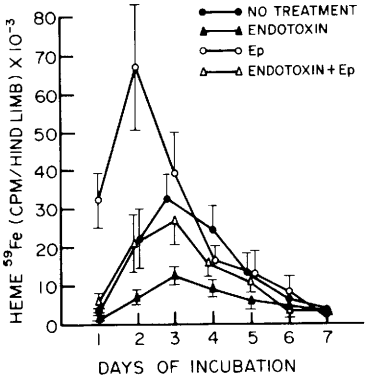


FIG. 2. Daily $^{59}$Fe incorporation into heme of cultured marrow cells from hypertransfused mice. After 4 days of treatment, nucleated cell counts × $10^{-6}$/hind limb were: no treatment, 18.1 ± 0.8; endotoxin 11.3 ± 0.9; Ep, 22.5 ± 0.6; endotoxin + Ep, 9.4 ± 1.4. Data represent mean ±SEM for five experiments except Ep and endotoxin + Ep groups which represent three experiments.

less prominent, but significant, increase over nontreated controls was noted in splenic CFU-E ($P < 0.001$) and Days 1 and 2 $^{59}$Fe incorporation ($P < 0.01$). When compared to nontreated mice, the marrow of the endotoxin group had decreased nucleated cells, CFU-E, and Day 3 BFU-E (all three $P$ values < 0.001) (Table I, Fig. 2). Marrow $^{59}$Fe incorporation was decreased from control levels on each day, but the greatest decrease was on Day 3 ($P < 0.05$).

In contrast, the spleens of Ep-treated mice, compared to those receiving no treatment, had a large increase in CFU-E and Days 1 and 2 $^{59}$Fe incorporation with a relatively smaller, but significant increase in total nucleated cells ($P < 0.05$), Day 3 BFU-E ($P < 0.01$), and Days 3 ($P < 0.01$) 4, and 5 ($P < 0.05$) $^{59}$Fe incorporation into heme (Table I, Fig. 1). In the marrow of Ep-treated mice, CFU-E ($P < 0.001$) and Days 1 and 2 $^{59}$Fe incorporation ($P < 0.02$) were much greater than those of nontreated controls, but marrow Day 3 BFU-E and Days 3–7 $^{59}$Fe incorporation were similar to controls (Table I, Fig. 2) while the number of total nucleated cells was

slightly increased. Compared to Ep-treated mice, the spleens of mice given endotoxin had less CFU-E ($P < 0.001$) and markedly less Day 1 $^{59}$Fe incorporation, yet they had very markedly increased Day 3 BFU-E and Days 3–5 $^{59}$Fe incorporation.

The spleens of mice given endotoxin + Ep had nucleated cell counts that were not significantly different from those given Ep, but their CFU-E and BFU-E ($P < 0.001$) and Days 2–4 $^{59}$Fe incorporations ($P < 0.01$) were greatly increased over those mice receiving Ep alone (Table I and Fig. 1). The splenic incorporation of $^{59}$Fe on Days 5–7 was similar in both groups (Fig. 1). Compared to the endotoxin group, the mice treated with endotoxin + Ep had much more splenic CFU-E and Days 1 and 2 $^{59}$Fe incorporation, with a relatively smaller but significant increase in nucleated cells ($P < 0.001$), but by 4 and 5 days of cell incubation the $^{59}$Fe incorporation was significantly decreased ($P < 0.05$) (Fig. 1). Compared to Ep-treated mice, the marrow of mice treated with endotoxin + Ep had markedly decreased nucleated cells and CFU-E, and decreased Days 1 and 2 $^{59}$Fe incorporation ($P < 0.01$). Marrow $^{59}$Fe incorporation on Days 3–7 was similar in the endotoxin + Ep group and the group receiving Ep alone (Fig. 2). The marrow of mice treated with endotoxin + Ep, when compared to endotoxin-treated mice, had nucleated cell counts that were similar, but the CFU-E and Day 3 BFU-E were increased ($P < 0.001$) and Days 1–4 $^{59}$Fe incorporation also was increased ($P < 0.05$) (Table I, Fig. 2).

*Discussion.* These studies used plethoric mice, which have decreased numbers of CFU-E (1), and concentrations of limulus-absorbed Ep *in vitro* which were suboptimal for Day 8 BFU-E development, but which allowed Day 3 BFU-E development. Under these conditions, the predominant erythroid progenitor cell assayed in mouse spleen and marrow was the Day 3 BFU-E. These BFU-E, however, are a heterogeneous population whose progeny first appear on Day 3 of culture and last through Day 5 (4). Such heterogeneity results in the continual appearance and subsequent senescence of the bursts, but these dynamic events are not assessed by the scoring of bursts which is performed at a fixed time. A parallel relation between serial CFU-E numbers and 4-hr $^{59}$Fe incorporation (3), and between Day 8 BFU-E and 24-hr $^{59}$Fe incorporation (17), previously has been demonstrated; and our data from Table I, when graphed per organ, show a linear relationship between Day 3 BFU-E and Day 3 $^{59}$Fe incorporation for both marrow and spleen. In our studies the numbers of CFU-E and Day 3 BFU-E correlated best with Day 1 and Day 3 $^{59}$Fe incorporation into heme. We used the latter method for measuring kinetic changes in erythropoiesis *in vitro* because we believe it better reflects the overall heterogeneity of the erythroid precursor cells and it allows evaluation of very late progenitors such as proerythroblasts, whose proliferative capacity is not sufficient to form colonies, but whose heme synthetic capacity is quite prominent. As we tried to assess total erythropoiesis, and as changes in the number of CFU-E and Day 3 BFU-E bore no constant relation to nucleated cell counts, all data were expressed per spleen or hind limb.

When endotoxin was administered to plethoric mice a marked increase in Day 3 BFU-E and smaller increases in CFU-E and Day 1 $^{59}$Fe incorporation were observed in the spleens. At the same time the marrow had a marked decline in the numbers of CFU-E and Day 3 BFU-E. These results are consistent with a shift of these cells from the marrow to the spleen as proposed for pluripotent colony-forming cells (CFU-S) (18). However, it is also possible that endotoxin increases the replication of Day 3 BFU-E in the spleen since it has been shown to increase the number of splenic CFU-S in cell cycle (19). In contrast to the effect of endotoxin, Ep produced a marked increase in CFU-E in both the marrow and spleens of these animals with only a very slight increase in splenic Day 3 BFU-E, and it had no significant effect on the number of marrow Day 3 BFU-E.

When endotoxin was administered with Ep and the effect was compared to the effect of Ep alone it is clear that endotoxin enhanced the magnitude of the Ep effect on the spleen, producing a further increase in the number of splenic CFU-E and an even greater increase in Day 3 BFU-E. Likewise, endotoxin greatly reduced the magnitude of the Ep ef-

fect on the marrow producing a marked decline in the number of CFU-E.

In contrast, the effect of Ep and endotoxin on the splenic cells compared to the effect of endotoxin alone, was to increase the number of CFU-E and decrease the number of precursor cells that gave rise to heme synthesis on Days 4 and 5. This decrease in less differentiated precursor cells was clearly revealed by the cumulative $^{59}$Fe incorporation studies whereas it was not evident in the $^{59}$Fe incorporation and count of BFU-E on Day 3. Without the addition of Ep, the large number of splenic Day 3 BFU-E induced by endotoxin showed little development into CFU-E, except what might be accounted for by small residual endogenous levels of Ep in plethoric mice. In the marrow, CFU-E, Day 3 BFU-E and Days 1–4 $^{59}$Fe incorporation were increased when Ep was given with endotoxin, but the shift to more differentiated progenitors was less than that seen in the spleen and no disproportionate decline of Days 4 and 5 $^{59}$Fe incorporation was evident.

These experiments indicate that endotoxin greatly enhances the number of Day 3 BFU-E in the spleens of plethoric mice, but it has little effect on erythropoietic differentiation. Ep, on the other hand, produces differentiation on erythroid progenitor cells and leads to an increased number of splenic CFU-E accompanied by a decline in the number of erythroid precursor cells that produce hemoglobin-synthesizing erythroblasts by Days 4 and 5 *in vitro*. These results extend the observations of Reissmann *et al.* (11) who postulated that the primary effect of endotoxin might be to increase the numbers of splenic erythroid precursor cells that preceded the CFU-E development. The only previous report of Ep causing an increase in CFU-E with an accompanying decrease in BFU-E is that of Iscove (20) who showed such a relation between CFU-E and Day 10 BFU-E in marrow of bled mice when the marrow was examined 4 days after bleeding. Our experiments do not exclude an effect of Ep on the self-replication of CFU-E, but they do suggest that the latter can be generated from developing BFU-E.

*Summary.* When the marrow and spleen cells of plethoric mice are cultured with 1.0 unit/ml of limulus-lysate-absorbed Ep, the predominant erythroid progenitor cell assayed is the Day 3 BFU-E. Erythropoiesis by these cells can be studied by enumeration of bursts at 3 days or by 24-hr $^{59}$Fe incorporation into heme. Endotoxin markedly increased the number of Day 3 BFU-E in the spleens of these mice and decreased the number in the marrow, but it had little effect on erythropoietic maturation. In contrast, Ep produced differentiation of the erythroid progenitor cells resulting in increased splenic CFU-E and a decline in the more primitive erythroid precursor cells.

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