Endotoxin-Induced Suppression of Erythropoiesis: The Role of Erythropoietin and a Heme Synthesis Stimulating Factor

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The regulation of erythropoiesis is primarily controlled by erythropoietin (Ep). Recently, however, other factors that both stimulate and inhibit erythropoiesis have been reported. Using an in vitro liquid culture of bone marrow cells, a factor in normal mouse serum was demonstrated that markedly stimulated heme synthesis by marrow erythroid cells. In this study, the role of this heme synthesis stimulating factor (HSF) and Ep in the erythropoietic suppression caused by endotoxin administration to mice was examined. Although HSF levels did not alter appreciably after endotoxin injection, marrow erythroid cells from these animals became unresponsive to the factor. This could be reversed if Ep was added to the culture in vitro or if the hormone was injected into the mice 18 hr prior to harvesting the marrow. This marrow erythroid cell response is identical to that seen in animals in whom Ep levels are markedly reduced, such as that found in exhypoxic polycythemia, and suggest a decrease in the hormone following endotoxin administration. Additional studies demonstrated that when Ep was injected into mice 8 hr after endotoxin administration, an increase in femoral erythroid colony-forming units (CFU-E), proerythroblast number, and 59Fe incorporation into femoral marrow cells could be demonstrated. These findings, together with the marrow erythroid cell response to the hormone, suggest that the mechanism for suppression of erythropoiesis after endotoxin injection is a reduction in the level of circulating Ep.

MATERIALS AND METHODS

Studies were performed on female CF1 mice (Charles River, Wilmington, Mass.) ranging in weight from 23–27 g. The mice were killed by cervical dislocation, and their femoral and tibial marrow was flushed in alpha medium containing 2% fetal calf serum (FCS, Flow Laboratories) using a 1-ml syringe fitted with a 23-gauge needle. Marrow cells were depleted of hemoglobin-containing erythroid cells as described previously and may be briefly summarized as follows. Total femoral cells were determined using a Coulter counter, and the cell suspension was then adjusted to 6 x 106 cells/ml. Guinea pig complement (0.05 ml/ml reaction volume) was then added dropwise with stirring. A predetermined dilution of anti-mouse red blood cell serum (0.1 ml) was added drop by drop, and the mixture was incubated for 15 min at room temperature with continuous stirring. The cell suspension was centrifuged at 250 g at 4°C for 5 min and the cell pellet was washed twice with alpha medium, and additions of heat-inactivated (56°C, 30 min) mouse serum and/or human urinary Ep dissolved in alpha medium. (Ep, pool H 32-TA SL, 27.8 U/mg, was kindly supplied by the Ep committee, National Heart, Lung and Blood Institute.) To control

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cultures, corresponding volumes of alpha medium were added. The cultures were divided into 3 aliquots of 0.5 ml and were incubated in a loose-capped tube for 24 hr at 37°C in humidified air containing 5% CO₂. At the end of this time, the cells were washed twice with alpha medium, prewarmed to 37°C, and were suspended in 0.5 ml alpha medium containing 1 μCi⁹⁹Fe preincubated with 25 μg human transferrin. Following a 3-hr incubation at 37°C, the cells were washed twice with phosphate-buffered saline and heme extracted using methylethyl ketone according to the method of Teale.⁹ The ⁹⁹Fe was then counted in a liquid scintillation counter (Packard, Downers Grove, Ill.) after adding 10 ml of liquid scintillation fluid. Previous studies have shown that the amount of radioactivity incorporated into heme is directly proportional to the degree of erythroblast stimulation.⁷ The results of each study were expressed as a percent of the control sample containing alpha medium. CFU-E was cultured in plasma clot by the method of McCleod et al.¹¹ Proerythroblasts were enumerated by performing a differential on stained marrow cell cytospin preparations following the criteria as described by Cantor et al.¹² and from the total femoral marrow cell number. Endotoxin (lipopolysaccharide B₃, S. typhosa, Difco) was diluted in normal saline and 20 μg injected i.p.

RESULTS

Since Ep and HSF together stimulate heme synthesis, an initial study was done to determine the dose of Ep which, in the presence of mouse serum, caused maximal enhancement of heme synthesis. When constant volumes of mouse serum and varying amounts of Ep were added to the culture medium, a proportional increase in ⁹⁹Fe incorporation into heme occurred (Fig. 1). This reached a maximum with an Ep dose of 400 mU, after which flat curves were noted. In a second study this concentration (400 mU) of Ep was used but the volume of mouse serum was varied from 1–200 μl. A proportional increase in ⁹⁹Fe incorporation into heme occurred that was linear when from 10–100 μl of mouse serum was added to the culture medium (Fig. 2). In this system, the addition of 400 mU Ep resulted in a ⁹⁹Fe incorporation into heme that ranged from 123 to 605 cpm, while the control sample ranged from 20 to 87 cpm. In every case, the addition of as little as 1 μl of mouse serum to the sample containing 400 mU Ep caused a further detectable and significant increase in the amount of ⁹⁹Fe incorporation into heme. The addition of 100 μl of normal mouse serum increased the radioactivity to a mean of 4674 cpm ranging from 4463 to 4979 cpm. The precision of the assay for HSF was derived by determining the within-assay variability of 6 control mouse sera, which averaged 7.3% of the mean and ranged from 6.0% to 8.4%. The between-assay variability of a single sample measured in 9 assays was 32% of the mean. Because of this variability in ⁹⁹Fe incorporation, which occurred when the same sample was measured on different days, the results of a test serum are always expressed as percent of a control serum.

In subsequent studies this assay was used to evaluate...
the effect of endotoxin injection on circulating HSF levels. Twenty micrograms of endotoxin were injected into 6 mice and the animals were killed 24 hr later. As shown later, at this time a significant reduction in marrow CFU-E and proerythroblast number can be demonstrated.6,7 Figure 3 shows a representative plot of ⁵⁹Fe incorporation into heme when serum collected after the injection of endotoxin was added to the culture medium. These values were compared to the heme synthesis when sera from normal animals were added to the culture. Endotoxin caused an insignificant decrease in circulating HSF levels, which averaged 85% of control and ranged from 69% to 98% of control. Serial dilutions of serum from endotoxin-treated animals were parallel to that of control animals, indicating the absence of inhibitors to serum-stimulated heme synthesis (Fig. 3). In subsequent studies, sera were collected from mice who had received endotoxin 3-48 hr previously, and at no time could a significant reduction in HSF levels be demonstrated.

Since injection of endotoxin causes a significant reduction in the number of marrow proerythroblasts, a study was then done to determine whether or not the marrow cells from endotoxin-treated mice could be stimulated when incubated with serum from control mice. In this study, 3 mice were injected with endotoxin and killed 24 hr later. Their marrow was repeatedly flushed as described above and treated with anti-mouse RBC serum to obtain a marrow cell preparation without hemoglobin containing erythroid cells. This was then cultured with and without the addition of normal mouse serum. Generally, when mouse serum is added to normal mouse marrow, a 20-30-fold increase in ⁵⁹Fe incorporation into heme can be demonstrated.8 In contrast, marrow from the mice injected with endotoxin did not show a significant increase in ⁵⁹Fe incorporation when compared to control cultures containing alpha medium (Table 1). However, when 200 mU Ep together with 100 μl of mouse serum was added to this marrow, a 20-fold increase in heme synthesis was noted (Table 1). This suggested that the major reason for the decrease in serum-stimulated heme synthesis was a lack of in vivo Ep activity or a reduction in its level. Additional studies were done on mice who received endotoxin and 6 hr later were injected with 2 U Ep. The marrow cells from these mice were cultured 18 hr later (24 hr after endotoxin injection) with and without normal mouse serum. Marrow cells from animals receiving endotoxin and Ep showed a 40-fold increase in ⁵⁹Fe incorporation into heme when 100 μl normal mouse serum was added to the culture medium. This response approximated that detected in marrow obtained from control animals. Furthermore, when Ep was added to the culture medium together with mouse serum, a further increase in ⁵⁹Fe incorporation into heme similar to that of control marrow was noted (Table 1).

Since the injection of 2 U Ep used in the above study was very large, the effect of injecting this dose into 3 normal mice 18 hr before sacrifice was examined. When their marrow cells were cultured in vitro, the increase in heme synthesis due to the addition of 0.1 ml of mouse serum averaged 1955% of the control, which was similar to an increase of 2016% seen in mice who did not receive an Ep injection.

Table 1. Effect of the Addition of Normal Mouse Serum With or Without Ep on Heme Synthesis in Cultures of Marrow Cells From Mice Injected with 20 μg Endotoxin i.p. 24 hr Earlier*

<table>
<thead>
<tr>
<th>Added to 1.5 ml Culture</th>
<th>No Ep Injection (cpm)</th>
<th>2 U Ep at 6 hr (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μl medium (control)</td>
<td>66 (±22)†</td>
<td>55 (±18)</td>
</tr>
<tr>
<td>100 μl mouse serum</td>
<td>107 (±27)</td>
<td>2,258 (±242)</td>
</tr>
<tr>
<td>100 μl mouse serum + 200 mU Ep</td>
<td>1,475 (±97)</td>
<td>4,405 (±557)</td>
</tr>
</tbody>
</table>

*Half the animals received 2 U Ep injection 6 hr after endotoxin. †Mean ± 1 SE.
In order to further delineate the correctability by Ep of the suppression in erythropoiesis following endotoxin administration further studies were done to evaluate femoral CFU-E and proerythroblast number. In this study, femoral CFU-E and proerythroblasts were quantitated on 5 control mice and 14 mice who had received 20 µg of endotoxin 24 hr previously. Seven of these latter animals received an additional injection of 2 U Ep 6 hr later. In 3 additional groups consisting of the same numbers of animals, 59Fe was injected i.p. 44 hr after the injection of endotoxin, and 4 hr later, the percent of injected radioactivity in the femoral marrow cells was determined. The injection of endotoxin resulted in significant reductions in femoral CFU-E and proerythroblast number (p < 0.001). The mean CFU-E was 77.9 x 10³/femur in the endotoxin-treated group compared to 197.4 x 10³ CFU-E/femur in the control group (Table 2). Corresponding values for proerythroblast number were 148.3 and 500.0 x 10³ cells/femur in the 2 groups, respectively. This decrease in erythropoiesis was associated with a significant reduction in the percent of 59Fe present in the marrow cells of the endotoxin-treated group (Table 2). The injection of 2 U Ep 6 hr after the injection of endotoxin appeared to substantially restore erythropoiesis. A significant increase in proerythroblast number and 59Fe incorporation into marrow cell was noted in the group that received Ep compared to those in which endotoxin alone was injected (Table 2). In addition, although not statistically significant, the mean CFU-E number was higher in the animals receiving Ep after endotoxin injection.

### DISCUSSION

Recently, using an in vitro liquid culture technique, factors affecting the stimulation of erythroid cells have been examined. Studies have shown that a marked stimulation of heme synthesis occurred by a factor or factors present in normal mouse serum (HSF) and by Ep. The effect of HSF is both greater than and independent of that of Ep. HSF activity is, however, intimately tied to that of Ep, so that when added together, a markedly synergistic enhancement of heme synthesis occurs. This intimate interrelationship between HSF and Ep makes it difficult to differentiate the effects of the former from that of the latter. For this reason, an assay was developed in which HSF activity alone could be examined. This was achieved by initially determining the dose of Ep that maximally stimulates heme synthesis. Once this was determined, the addition of mouse serum caused a further dose-related increase in heme synthesis. Although the method was relatively precise when multiple samples were examined in the same assay, day-to-day variation was wide, and therefore, results of a test serum were always compared to that of a control serum. The studies of Krystal et al. indicate that serum may contain multiple heme synthesis enhancing factors, at least one of which is labile with storage. This fact could account for the day-to-day variability seen in our assay.

Previous studies have examined the effect of HSF on marrow erythroid cells obtained from exhypoxic polycythemic mice in whom Ep levels are markedly suppressed. Marrow erythroid cells from those animals only respond to HSF if Ep is simultaneously added to the culture medium or if Ep is injected into the mouse at least 18 hr before obtaining the marrow. These results led to the hypothesis that erythroblasts have to be initially primed by Ep before becoming responsive to HSF. The present study indicated that the injection of endotoxin into mice caused changes in erythroblasts that mimic those found in the exhypoxic polycythemic mouse system. Thus, they only responded to HSF when Ep was added to the culture in vitro or if the hormone was previously injected into the animal. These findings, coupled with the fact that HSF levels were not reduced in endotoxin-treated mice, suggest that the major abnormality accounting for the effect on serum-stimulated heme synthesis could be the neutralization of Ep production after endotoxin injection. In normal animals, the injection of a large dose of Ep 18 hr prior to study does not increase femoral CFU-E or proerythroblast response to HSF. In contrast, the injection of 2 U Ep into endotoxin-treated animals 18 hr before sacrifice largely minimized the erythropoietic suppression that endotoxin causes. This is also consistent with the hypothesis that a lack of the

### Table 2. Effect of 20 µg Endotoxin Injection to Mice With or Without the Injection of 2 U Ep 6 hr Later.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>CFU-E</th>
<th>Proerythroblast</th>
<th>48 hr After Injection of 2 U Ep 6 hr later</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>197.4 ± 25.1</td>
<td>500.0 ± 68.0</td>
<td>1.09 ± 0.19</td>
</tr>
<tr>
<td>20 µg endotoxin</td>
<td>7</td>
<td>77.9 ± 16.1</td>
<td>148.3 ± 28.3</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>20 µg endotoxin +</td>
<td>7</td>
<td>112.6 ± 28.3</td>
<td>321.3 ± 78.3</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>2 U Ep 6 hr later</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Femoral CFU-E, proerythroblast number, and marrow cell 59Fe incorporation are shown.
†Mean (± 1 SE).
circulating hormone may be the major mechanism accounting for the reduced erythropoiesis. Not only could an enhanced stimulation of heme synthesis be demonstrated, but increases in femoral CFU-E and proerythroblast number were also found, as was a greater uptake of $^{59}$Fe into the femoral marrow cells of animals receiving Ep after the endotoxin injection. The studies of Schade and Fried, who demonstrated a reduction in renal Ep production after endotoxin injection, also support our findings. It must be noted that a single injection of Ep did not completely correct the defect. One possibility relates to the transient nature of a single injection compared to the continuous in vivo effect of the hormone. It also remains possible that other mechanisms contribute to the reduced erythropoiesis. Recent evidence suggests that endotoxin administration may result in BFU-E suppression caused by a factor contained in marrow-adherent cells. Thus, a decrease in delivery of cells from the BFU-E to CFU-E compartments could account for some suppression in erythropoiesis. Circulating inhibitors to erythropoiesis have also been proposed. However, in this study, serial dilutions of serum from control and endotoxin-treated mice resulted in parallel increases in heme synthesis, making the presence of an inhibitor unlikely. An additional possibility is that the reduction in CFU-E, proerythroblast number, and marrow $^{59}$Fe uptake is caused by the egress of hematopoietic cells from the marrow, which has been described after endotoxin injection. If this were the major mechanism of reduced erythropoiesis, an increase in CFU-E and proerythroblast number after the injection of Ep in vivo would not be expected. For similar reasons these observations also do not support the postulate that erythropoietic suppression after endotoxin administration is due to the preferential utilization of bone marrow stem cells for granulopoiesis.

REFERENCES

3. Golde DW, Bersch N, Quan SG, Lusis AJ: Production of erythroid potentiating activity by a human T-lymphoblast cell line. Proc Natl Acad Sci USA 77:593, 1980
Endotoxin-induced suppression of erythropoiesis: the role of erythropoietin and a heme synthesis stimulating factor

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