PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 98-101 (1976)

Induction of Erythroid Colony Forming Cells (CFU-E) in Murine Spleen by Endotoxin (39488)[1]

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The plasma clot culture system containing erythropoietin (Ep) permits erythroid precursors from bone marrow or spleen to form *in vitro* small colonies of hemoglobin-synthesizing erythroblasts (1). Early erythroid precursors (committed erythroid stem cells) most likely comprise a multistage compartment that is interposed between the pluripotential stem cells (CFU) and the morphologically recognizable proerythroblasts (2, 3). The position of the colony-forming erythroid precursors (CFU-E) within this compartment and the factors regulating or influencing their population size are not exactly known. Erythropoietin injection into hypertransfused mice has been shown by Gregory *et al.* (4) to increase the number of femoral or splenic CFU-E, but increasing the concentration of Ep in the medium above a plateau level failed to increase colony formation. The injection of endotoxin into mice is known to increase their splenic erythropoiesis as measured by ^{59}Fe incorporation (5, 6), and the present study was undertaken to examine the effect of endotoxin on the number of splenic CFU-E in the presence or absence of Ep. It will be shown that endotoxin injection indeed induced splenic CFU-E in normal mice and in mice whose endogenous erythropoietin production was suppressed by posthypoxic polycythemia. Differences in increases in CFU-E between those induced by Ep and those induced by endotoxin will be discussed.

Methods. Female CF_1 mice of from 23 to 27 g body weight were used in groups of eight. Mice were made polycythemic by 3 weeks of exposure (18 hr/day) to an atmospheric pressure of from 380 to 340 mm Hg. Endotoxin (lipopolysaccharide B, *S. typhosa,* Difco) was diluted in normal saline and injected ip. Human erythropoietin (56 units/mg) was obtained through the Erythropoietin Committee of the NIH Heart-Lung Institute. The Ep was processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles. The Ep was dissolved in 0.94 g% sodium chloride solution and injected iv in doses as indicated.

For CFU-E measurement, spleens were removed at intervals as indicated, after the last injection of either Ep or endotoxin. The spleen was cut in small pieces and a cell suspension was prepared in supplemented Eagle's MEM (Grand Island Biological Company) by drawing the cells back and forth through a 19-gauge and then a 20-gauge needle. An aliquot was used for cell counting in a Coulter Counter using cetremide (10) for lysis of erythrocytes. The number of spleen CFU-E was measured by a modification of the 2-day plasma clot culture method of McLeod *et al.* (1). The culture medium consisted of 2 ml of alpha medium (Flow Laboratory, Rockville, Md.), 0.1 ml of a 10^{-3} *M* solution of alpha thioglycerol (9), 1.5 ml of heat-inactivated (30 min, 60°) fetal calf serum (GIBCO), 0.25 ml of detoxified (1) bovine serum albumin solution (20%), 0.055 ml of L-asparagine solution (2 mg/ml), 0.17 ml of beef embryo extract (GIBCO), and 0.5 units of Ep (dissolved in alpha medium) per ml of medium. A measured aliquot of each spleen cell suspension was added to a total volume of 1.5 ml of medium and was plated in 12 microwells (0.1 ml/well). The cell count ranged from eight to ten 10^5 per milliliter of medium. After incubation for 48 hr at 37° in air plus 5% CO_2, the clots were then transferred to slides, stained with benzidine-hematoxylin, and colonies of eight or more benzidine positive cells were scored.

In some experiments a portion of the cell suspension ($3 \cdot 10^6$ cells) was transferred on slides by means of cytospin centrifuge. The preparations were stained with Giemsa and the presence of proerythroblasts was scored

[1] This study was supported by USPHS Grant AM 07239.

from 0 to +4 by two observers.

The splenic ^{59}Fe incorporation into heme was measured by iv injection of 1.5 μCi of Fe^{59} 48 hr after Ep or the last endotoxin injection. Six hours later a cell suspension of the spleen was made as described above. The cell suspension was washed in saline and the cells were ruptured through repeated freezing and thawing. One milliliter of Drabkin's solution was then added and after standing overnight the stromata were removed by high speed centrifugation. The pH was then adjusted to 2.0 and the heme was extracted by shaking with methyl ethyl ketone. Results are expressed as the percentage of the injected ^{59}Fe present in the extracted heme. The Student's *t* distribution was used to assess statistical significance.

Results. The number of CFU-E per spleen ranged from 82 to $110 \cdot 10^3$ in normal mice and from 21 to $46 \cdot 10^3$ in polycythemic mice on the sixth posthypoxic day. Table I shows the effects of a single injection of from 5 to 50 μg of endotoxin on splenic CFU-E plated 72 hr later and on the 6-hr splenic ^{59}Fe incorporation into heme measured 96 hr after endotoxin injection. Ten micrograms or more induced significant ($P < 0.05$) increases in CFU-E, whereas the percentile increase in the *in vivo* ^{59}Fe incorporation was much smaller and was only significant after injection of 50 μg of endotoxin. No erythroid colonies developed when spleen cells from normal or from endotoxin-treated mice were cultured without Ep in the culture medium.

Table II shows the progressive increase in splenic CFU-E in normal and in polycythemic (6 days posthypoxia) mice after 2, 4, and 6 days of injection of 10 μg of endotoxin per day. In the normal mice a 40-fold increase in CFU-E was found after six injections, and a 21-fold increase was found in the polycythemic mice. The absolute number of CFU-E in the endotoxin-treated polycythemic mice was more than eight times greater than that in untreated normal, i.e., nonpolycythemic, mice. The splenic ^{59}Fe incorporation was markedly different in the two groups. The progressive rise in splenic iron incorporation seen with endotoxin treatment in the nonpolycythemic mice was absent in the mice whose endogenous production of erythropoietin had been suppressed by polycythemia. Injection of 1 unit of erythropoietin 1 day after termination of the six endotoxin injections induced an increase in splenic ^{59}Fe incorporation from 0.027 to 0.86%, indicating that the endotoxin-induced precursors were capable of *in vivo* development into heme-synthesizing erythroblasts. In the absence of erythropoietin, however, the endotoxin-induced precursors were not transformed *in vivo* into morphologically identifiable proerythroblasts. In the experiment presented in Table III, cytospin preparations of spleen cell suspensions were examined for the presence of proerythroblasts at various times after injection of Ep or of endotoxin into posthypoxic polycythemic mice. Very few proerythroblasts were seen in the spleens of polycythemic mice on the fifth posthypoxic day, and no significant increases occurred after endotoxin injection. Ep, in contrast, induced within 16 to 24 hr a sizable cohort of splenic proerythroblasts.

Table III also shows a significant differ-

TABLE II. EFFECT OF DAILY INJECTIONS OF 10 μg OF ENDOTOXIN ON CFU-E AND 6-HR ^{59}Fe HEME INCORPORATION IN SPLEENS OF NORMAL AND POLYCYTHEMIC MICE[a]

Days of endotoxin	Normals		Polycythemic	
	CFU-E $\cdot 10^{-3}$	^{59}Fe (%)	CFU-E $\cdot 10^{-3}$	^{59}Fe (%)
0	85	1.04	34	0.013
2	459	2.23	186	0.019*
4	1554	3.44	472	0.014*
6	3997	4.53	715	0.021*

[a] Eight mice per group.
* Not significant ($P > 0.05$).

TABLE I. EFFECT OF ENDOTOXIN DOSE ON CFU-E AND 6-HR ^{59}Fe HEME INCORPORATION IN SPLEENS OF NORMAL MICE[a]

	Endotoxin dose (μg)				
	0	5	10	30	50
CFU-E $\cdot 10^{-3}$	102 ± 21	188 ± 38	272 ± 38	397 ± 97	467 ± 121
^{59}Fe in heme (%)	1.04 ± 0.23	0.96 ± 0.06	1.07 ± 0.31	1.58 ± 0.25	2.17 ± 0.41

[a] Eight mice per group; mean ± SEM.

TABLE III. EFFECT OF A SINGLE INJECTION OF 30 μg OF ENDOTOXIN OR OF 1.5 UNITS OF ERYTHROPOIETIN ON CFU-E AND PROERYTHROBLASTS (PE) IN SPLEENS OF POLYCYTHEMIC MICE[a]

Time of injection before culture (hr)	Erythropoietin		Endotoxin	
	CFU-E[b] · 10^{-3}	PE	CFU-E[b] · 10^{-3}	PE
0	38 ± 13	±	38 ± 13	±
16	156 ± 50	++	51 ± 10	±
24	154 ± 23	++++	87 ± 25	±
48	102 ± 22	+++	162 ± 25	±
72	32 ± 12	±	201 ± 21	±
96	30 ± 16	±	249 ± 36	±
120	34 ± 17	±	79 ± 21	±

[a] Eight mice per group.
[b] Mean ± SEM.

ence between Ep and endotoxin in the time course of CFU-E induction. Ep injection resulted in a maximal increase at 16 hr after its injection, and the CFU-E returned to base line levels at 72 hr after Ep. In contrast, no significant splenic CFU-E increases were found at 16 or 24 hr after endotoxin, and maximal increases occurred between 72 and 96 hr after its injection.

Discussion. The presented data show that endotoxin induced in the spleen of normal or of polycythemic mice erythroid precursor cells which differentiated *in vitro* in the presence of Ep into colonies of hemoglobin-containing erythroblasts. When left in the intact mouse whose endogenous Ep was suppressed by polycythemia, these precursors did not differentiate into identifiable proerythroblasts or later stages. These findings present clear confirmation of the capability of the plasma clot culture system to induce *in vitro* differentiation of erythroid precursors. In the nonpolycythemic mouse, the endotoxin-induced precursors differentiated *in vivo*, and this resulted in increases in splenic ^{59}Fe incorporation into heme. Injection of Ep into endotoxin-treated polycythemic mice achieved the same effect. The induction of splenic CFU-E by endotoxin thus did not require the presence of Ep, but the transformation of CFU-E into erythroblasts required its presence both *in vivo* and *in vitro*.

The number of splenic CFU-E in polycythemic mice could also be markedly increased by an injection of Ep, and our reults thus confirm earlier findings of Gregory (4). The different time element in the induction of CFU-E by endotoxin versus that by Ep suggests different modes of action. Maximal CFU-E increases occurred from 72 to 96 hr after injection of endotoxin. Endotoxin has been shown to turn noncycling CFU into cell cycle (7) and to increase the number of splenic CFU (8). It would seem plausible, therefore, that the relative late increase in CFU-E was the result of a proliferation-stimulating effect of endotoxin on either the pluripotential or the committed erythroid stem cells. Erythropoietin, in contrast, induced maximal CFU-E numbers within 16 hr of its injection. A number of observations (5, 6) indicates that Ep not only causes *in vivo* transformation of precursors (ERC) into proerythroblasts, but that it enhances at the same time the development of earlier precursors into ERC. The egress of ERC through their erythropoietin-regulated transformation into proerythroblasts is thus balanced, and the population size of ERC is maintained at various rates of erythropoiesis. It is reasonable to assume that the CFU-E are identical or closely related to the ERC, which are regarded to represent *in vivo* the immediate precursors of proerythroblasts. A single injection of Ep in the polycythemic mice increased the splenic CFU-E within 16 hr by 400%. This increase probably is the result of a recruitment of earlier precursors and it thus corresponds to the recruitment of immediate proerythroblast precursors (ERC) observed in the intact mouse. However, in view of the rather large increase occurring within a time span of 16 hr, an alternative explanation can be entertained, namely, erythroid colony formation by proerythroblasts in addition to the colonies formed by their immediate precursors. As seen in Table III, the marked CFU-E increase measured *in vitro* was accompanied *in vivo* by the appearance in the spleen of a sizable cohort of proerythroblasts. If the latter are also capable of forming colonies of erythroblasts *in vitro*, some of the CFU-E increases after Ep injection, and conversely some of the decreases in CFU-E in the post-hypoxic polycythemic mouse, would actually be attributable to changes in the number of proerythroblasts present at the time of obtaining spleen or marrow for culture. This possibility has a bearing on the inter-

pretation of *in vitro* colony-forming CFU-E as a measure of erythroid precursors and requires further investigation.

Summary. Injection of endotoxin induced in the spleens of normal and of polycythemic mice a dose-related increase in CFU-E. In the intact normal mice these precursors developed into erythroblasts and resulted in increases in ^{59}Fe incorporation into heme. In mice whose endogenous Ep was suppressed by polycythemia no differentiation of the precursors took place. The time course of the CFU-E increase after endotoxin suggests proliferation of precursors as the most plausible mechanism.

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Received May 7, 1976. P.S.E.B.M. 1976, Vol. 153.