Homogeneity of Erythropoietin in Its Effect on Self-Replication and Differentiation of Erythropoietin-Responsive Cells[1] (36769)

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It is well established that erythropoietin (Ep) regulates erythropoiesis by inducing differentiation of morphologically unrecognizable precursors (erythropoietin-responsive cells, ERC) into proerythroblasts (PE). More recently, evidence has been accumulating in support of a second action of Ep, namely stimulation of ERC proliferation and self-replication. In split-dose experiments (1, 2) the first dose of Ep was found to increase the erythropoietic response to the second dose, indicating that the former augmented the number or the responsiveness of ERC. Results of labeling of immediate PE precursors by tritiated thymidine suggested increases in their proliferation during accelerated erythropoiesis or after Ep injection (3, 4), and the same was evident in experiments employing hydroxyurea as a cycle-dependent inhibitor of ERC proliferation (5). Further evidence of a marked stimulation of ERC self-replication by Ep was obtained in mice whose pluripotent stem cells (CFU) and ERC had been severely suppressed by Myleran (6–8). In the absence of Ep, no ERC could be detected for periods up to 2 wk, but injection of large doses of Ep restored measurable ERC populations within a few days without enhancing CFU regeneration. The Ep-induced regeneration of ERC was sensitive to inhibition by hydroxyurea, and it is attributed to stimulation of ERC self-replication by the injected Ep. The latter thus appears to trigger some ERC into self-replication while inducing others into differentiation, and the question arises whether these two actions are exerted by one or by two biological entities. Completely purified and homogenous Ep is not available, and the problem was therefore approached in the present study by measuring the dose–response of 5 Ep preparations in regard to their effects on ERC self-replication and differentiation. The Eps were prepared by different methods and were of different origin and purity. If the two described Ep effects were exerted by two different biological entities, their presence in exactly the same proportions in the various Ep preparations would be unlikely, and in that case differences between the dose–response in regard to the two processes should be demonstrable.

Materials and Methods. Ep preparation A and B were obtained through the Erythropoietin Committee of the National Heart and Lung Institute. They were collected and concentrated by the Department of Physiology, University of Corrientes, Argentina, and were further processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles. Ep A was prepared by benzoic acid absorption (9) from the urine of anemic patients. It was purified by combined gel exclusion and anion exchange chromatography on QAE-Sephadex A50, and it contained 0.98 mg protein/mg. Ep B was prepared by collodion absorption from the urine of anemic patients. It was purified by Sephadex G-25 gel chromatography. Ep C was prepared in our laboratory from the urine of a patient with aplastic anemia by dialysis against Carbowax M20 (Union Carbide Corp.), followed by lyophilization. Ep D (purchased from Connaught Med. Lab. in Toronto) was step 1 Ep prepared from plasma of phenylhydrazine-treated sheep (10). Ep E was prepared in our laboratory from the urine of a patient with longstanding pure erythroid hypoplasia. The urine was dialyzed against Carbowax

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TABLE I. Effect of Ep A, B, C and D on Restoration of ERC Populations in Myleran-Suppressed Polycythemic Mice.[a]

Ep	3 × 8 units Ep	3 × 4 units Ep	3 × 2 units Ep	3 × 1 unit Ep
A	29.7 ± 2.5	22.1 ± 2.1	12.2 ± 2.5	7.9 ± 2.8
B	28.6 ± 1.8	22.5 ± 2.9	13.5 ± 3.7	
C	28.8 ± 1.8	20.3 ± 1.6	12.9 ± 2.0	6.2 ± 2.0
D	27.7 ± 1.5	20.1 ± 2.2	14.8 ± 3.4	8.5 ± 3.3

[a] Mean and SEM of percentage ^{59}Fe incorporation in groups of 8 mice.

M20 and water and then lyophilized. A fraction of it was purified by Sephadex G-75 gel chromatography. The reference Ep Standard B was kindly supplied by the British Medical Research Council, London.

The conventional ERC differentiation-inducing effect of Ep was measured in groups of 8 posthypoxic polycythemic mice. Female mice (20–25 g) were exposed 18 hr/day for 3 wk in a decompression chamber at from 380 to 350 mm Hg ambient pressure. On Day 4 after return to normal ambient pressure, each mouse received one sc injection of Ep or of the Ep standard. Two days later, 0.5 μCi ^{59}Fe was injected via tail vein, and 48 hr thereafter the radioactivity was counted on washed RBC of 0.2 ml heart blood. Blood volume was assumed to be 7% of body weight. All preparations and the standard were tested at 3 dose levels.

The effect of Ep on ERC proliferation was tested in groups of 8 posthypoxic polycythemic mice which received 44 mg of Myleran/kg body weight by stomach tube on Day 3 after return to normal ambient pressure. On Days 1, 2, and 3 after Myleran each mouse received sc either 8, 4, 2 or 1 units of the various Ep preparations. All mice were given on Day 5 after Myleran one sc injection of 1 unit of Ep A. The wave of erythropoiesis induced by this injection was measured by injecting 0.5 μCi ^{59}Fe on Day 7 after Myleran and by counting the radioactivity on washed RBC of 0.2 heart blood drawn 24 hr after the ^{59}Fe injection. Mice with hematocrits below 58% were discarded. Blood volume was assumed to be 7% of body weight.

In the inactivation experiments, 75 units of Ep was dissolved in 3 ml normal saline and incubated for 1 hr at 37° after adding 500 units of neuraminidase (Nutritional Biochemicals).

Results. The potency of the 5 Ep preparations as determined by comparison with Standard B in the posthypoxic polycythemic mouse assay were as follows: 161 units/mg Ep A, 45 units/mg Ep B, 2.8 units/mg Ep C, 0.38 units/mg Ep D and 0.78 units/mg Ep E. On logarithmic plots, all 5 Ep preparations showed linear and very nearly parallel dose/response curves.

The results of the measurement of their stimulatory effect on ERC replication in the Myleran-suppressed mouse assay are presented in Table I. Preparations A, B, C and D induced nearly identical ERC populations as measured by ^{59}Fe incorporation, and a clearcut dose–response relationship is evident. Ep E resulted at all dose levels in a significantly lower response, and the efficacy of 8 units Ep E was actually less than that of 4 units. This suggested the presence of a toxic or inhibitory substance whose suppressing effect was more pronounced at higher doses. After further purification by Sephadex G-75 gel chromatography, the potency of the Ep E had increased from 0.78 to 4.8 units/mg and it exerted now a stimulatory effect upon ERC self-replication which was similar to that of the other 4 preparations (Table II).

Incubation with neuraminidase abolished in all preparations both the differentiation-inducing and the ERC proliferation-stimulating effect. Table III shows as an example the values obtained before and after inactivation of Ep A.

Discussion. The magnitude of a wave of erythropoiesis generated by a single injection of Ep in a polycythemic mouse depends on the dose of Ep and on the size of the target

TABLE II. Effect of Ep E Before and After Purification on Restoration of ERC Populations in Myleran-Suppressed Polycythemic Mice.[a]

	3 × 8 units Ep	3 × 4 units Ep	3 × 2 units Ep
Before	11.9 ± 3.8	13.8 ± 2.8	7.6 ± 2.9
After	26.3 ± 2.2	21.0 ± 2.4	16.3 ± 3.9

[a] Mean and SEM of percentage ^{59}Fe incorporation in groups of 8 mice.

ERC population. The latter is fairly uniform in posthypoxic polycythemic mice, and the erythropoiesis, measured by means of ^{59}Fe incorporation, after an injection of Ep is thus a measure of the Ep dose or more precisely of its ERC differentiation-inducing action. If, on the other hand, a constant dose of Ep is given to mice whose ERC populations vary in size, the resulting erythropoiesis serves as a measure of the ERC populations (11), and this principle was used in assessing the ERC populations generated in Myleran-treated mice by large doses of Ep. As it has been described in detail elsewhere (6–8) the Ep injections given on the first 3 days after Myleran do not induce a wave of erythroblasts, but they do produce ERC populations which are then capable of responding with erythroblast formation to an injection of Ep given on Day 5 or later after Myleran. The ^{59}Fe incorporations in these mice are thus a measure of the ERC populations which were generated by the Ep injections given during the first 3 days after Myleran. This process required much larger Ep doses than the induction of ERC differentiation but a dose/response relationship is clearly evident. By comparing the response to various Ep preparations in the 2 assays, one can thus ascertain whether the agent that induces ERC differentiation and that which stimulates ERC proliferation were present in the preparations in identical proportions. This was indeed the case in spite of wide differences in origin, purity and methods employed in the preparation of the Eps, and it appears highly probable, therefore, that the two processes are regulated by one and the same biological entity. The decision as to which of the 2 processes is switched on by Ep in an individual ERC must rest with the cell itself. We have earlier presented evidence of an age structure within the ERC population (7), and it appears likely that the more mature ERC are induced into differentiation by Ep while less mature stages are stimulated by it into self-replication. During accelerated erythropoiesis a greater number of ERC is forced into differentiation, and if the ERC population is to be sustained, this greater cell outflow must be balanced either by an increased inflow through partial differentiation of CFU into ERC or by increased self-replication of the latter. The dual effect of Ep thus represents at least some degree of an autoregulation in the sense that the same agent which increases outflow of ERC through differentiation also provides the signal for an increased proliferation within the ERC population.

TABLE III. Effect of Inactivation of Ep A by Neuraminidase on ERC Differentiation (Column D) and Proliferation (Column P).[a]

	D 1 × 0.45 units Ep	P 3 × 4 units Ep
No inactivation	19.9 ± 2.1	20.8 ± 3.1
After inactivation	0.3 ± 0.2	0.2 ± 0.2

[a] Mean and SEM of percentage ^{59}Fe incorporation in groups of 8 mice.

The presence of an inhibitory substance in the Ep prepared from the urine of a patient with pure erythroid hypoplasia was not detectable when the ERC differentiation-inducing action of Ep E was tested in the conventional Ep assay, but it was clearly evident in the assay measuring the Ep effect on ERC proliferation. The inhibitory substance could easily be separated by Sephadex gel chromatography, and upon retesting, the purified Ep E exerted a stimulatory effect on ERC proliferation which was entirely com-

parable to that of the other Eps. The nature and particularly the specificity of the inhibitor are under investigation.

Summary. The ERC differentiation-inducing and the ERC proliferation-stimulating effects of 5 erythropoietin preparations were examined to determine whether the two effects were exerted by one or two biological entities. The Ep preparations included human and sheep Ep and ranged in purity from 161 to 0.38 units/mg. Injections at 4 dose levels in Myleran-suppressed mice failed to reveal any differences in the capability of the 5 preparations to stimulate ERC proliferation indicating that the two effects are very likely exerted by the same hormone.

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