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Effect of Erythropoietin on Human Bone Marrow Cells *in Vitro*. III. Studies of Acute Leukemia (38172)

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One of the most characteristic symptoms of acute leukemia is a severe and rapidly progressive anemia in the absence of massive hemorrhage or excessive hemolysis. In such cases, it is suggested that the bone marrow cells cannot respond to the increased concentration of erythropoietin (EP) induced by anemia (1, 2), or that the erythropoietic tissue is decreased in hemopoietic organs of the leukemic patient (3). From this point of view, it seems very important to know the responsiveness of hemopoietic cells to EP in leukemic patients.

For this purpose, we cultured bone marrow cells from patients with acute leukemia in the presence of exogenous erythropoietin by the method described before (4).

Materials and Methods. Five normal control subjects without any remarkable hematological disorders, 5 untreated patients with acute myelogenous leukemia (AML) and 5 untreated patients with actute lymphocytic leukemia (ALL) were the subjects in this study. The diagnosis of AML or ALL was based on the finding of peripheral blood and bone marrow film. Additional evidences for the diagnosis of AML and ALL were peroxidase and PAS reaction and electronmicroscopic observations of leukemic cells.

Table I summarizes the hematological data of the 10 cases of acute leukemia of which the bone marrow cells were cultured.

The bone marrow cells were cultured in the presence of EP by a modified method of Krantz and others (4, 5). In brief, human bone marrow cells aspirated by sternal puncture were washed twice with cold NCTC 109 solution (Difco) and suspended in an incubation medium consisting of 60% NCTC 109 solution, 40% inactivated human plasma of AB type, plus 50 U/ml of penicillin. The plasma was obtained from hematologically normal volunteers.

Eight tenths of an ml of a bone marrow cell suspension which had been previously adjusted with incubation medium to a cellularity of from 2000 to 4500 nucleated cells per mm^3 was placed into a 35 × 10 mm plastic tissue culture dish (Falcon plastics) and then in the presence of EP in a humidified atmosphere consisting of 5% CO_2 in air at 37° for period of 72 hr. The EP was obtained from the urine of a patient with aplastic anemia by means of 80% acetone precipitation and ammonium sulfate fractionation. The lyophilized EP preparation had a potency of 18.2 U/mg dry weight calibrated in starved rat assay against Erythropoietin Standard A. The EP preparation was dissolved in NCTC 109 solution and sterilized by filtering through a Millipore filter prior to use.

Six hours prior to the termination of incubation, 3 μCi of radioactive iron ($^{59}FeCl_3$, specific activity 4.5 mCi/mg diluted in HCl), preincubated with 0.1 ml of type AB plasma, was added to each dish. At the conclusion of incubation, the cells were washed 3 times with cold buffered saline, lysed with 1 ml of cold distilled water and added with 1 ml of cold 5 times concentrated Drabkin's solution. The heme was extracted from the lysate with 3 ml of acid

TABLE I. Hematological Data and Heme Synthesis Rate.

Name	Diagnosis	Peripheral blood: Hb (g/dl)	Peripheral blood: RBC ($\times 10^4$/mm^3)	Bone marrow: Retics (%)	Bone marrow: % Leukemic cells	EP (+) /EP (−) **: % Erythroblast*	EP (+) /EP (−) **: % Heme Synthesis rate
M.T.	AML	7.6	250	0	93.6	2.4	174
S.O.	"	6.7	268	0.9	93.5	0	133
K.Y.	"	4.6	115	0	49.0	0	123
Y.T.	"	7.6	206	0	70.8	4.0	206
H.I.	"	10.0	335	0.1	90.8	0.6	100
T.S.	ALL	7.1	235	3.6	91.2	4.2	2248
T.D.	"	14.8	491	0	94.2	1.5	977
N.H.	"	9.4	271	0.9	92.4	3.2	382
A.S.	"	8.1	235	2.0	94.5	5.0	761
S.Y.	"	4.5	113	1.2	96.0	0.8	518
Non-leukemic controls***		14.9 ± 0.76	486 ± 33	1.7 ± 0.4		28.5 ± 1.7	622 ± 58

* 0 represents erythroblasts could not be found among 1000 nucleated cells.

** $\frac{\text{cpm in dish containing 0.1 U/ml of erythropoietin}}{\text{" " without erythropoietin}}$

*** The average and a standard error of 5 cases without prominent hematological disorders. Cultures were done in triplicate.

methylethylketone as described previously (6). An aliquot of the methylethylketone layer containing heme was dried on a steel planchet and its radioactivity was counted in a gas-flow Geiger counter. Each culture was done in triplicate. The relative heme synthesis rate was expressed as the percentage of the radioactivity in a control culture without EP.

Results. When the marrow cells from hematologically normal subjects were cultured, the rate of heme synthesis was increased approximately 600% of the control levels by adding 0.1 U/ml of EP in the 5 cases studied (Table I). This value is very similar to those obtained by Krantz (7). Addition of EP to normal marrow cells up to 0.2 U/ml produced a linear increase in heme synthesis with a log dose of EP (Fig. 1). Figure 2 shows time course of a typical case of normal subject.

The response to 0.1 U/ml of EP in the cultures from hematologically normal subjects and patients with acute leukemia are summarized in Table I.

In all cases of AML, the response to EP after 72 hr culture was apparently lower than that in 5 normal subjects. On the contrary in 5 cases of ALL, the response to EP was maintained at normal levels, or even slightly above.

Discussion. In the present study, bone marrow specimen taken from all the cases of acute leukemia prior to treatment was intensively infiltrated with leukemic cells. Erythroblasts were reduced to a level less than 5%.

In all the cases of AML, the response to EP, expressed as relative heme synthesis rate, was markedly reduced. On the contrary, it is to be noted that in cases of ALL, the response was maintained even though the number of erythroblasts were drastically reduced at the beginning of culture.

In this experimental system, the heme synthesis rate at 72 hr of culture represents the response of erythropoietin-responsive cells (ERC's) to EP present at the beginning of culture or developed from multipotent stem cells during the culture period (4, 5), plus the response of erythroblasts already present at the beginning and still remaining to synthesize hemoglobin at the end of culture. The level of heme synthesis in the unstimulated culture may correspond

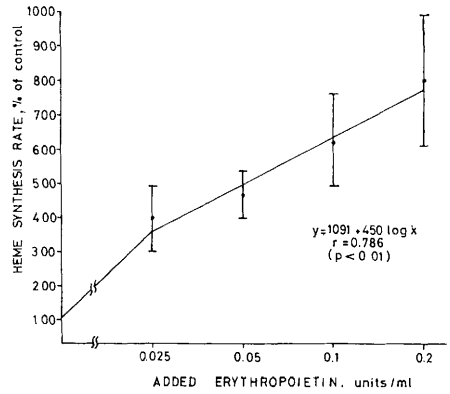

FIG. 1. Erythropoietin dose-response curve of human marrow cells *in vitro* obtained from 5 hematologically normal subjects. 2000 nucleated cells/mm^3 were present in each dish with 40% homologous AB plasma. After 66 hours of incubation 3 μCi of ^{59}Fe was added to each dish and culture was terminated 6 hr later. Each bar shows mean and its standard deviation of heme synthesis rate at each EP concentration. Correlation coefficient r and regression line are calculated from these values and are significant when $P < 0.01$.

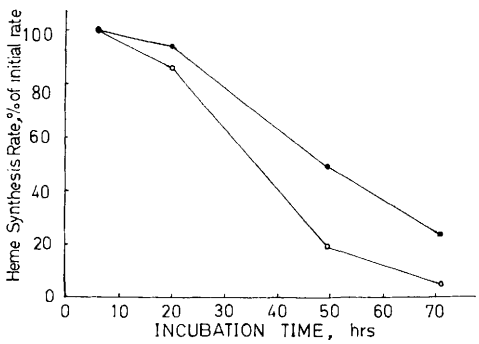

FIG. 2. The effect of duration of *in vitro* incubation on normal human marrow cells with and without added erythropoietin. In each dish 2000 nucleated cells/mm^3 were present with 40% homologous AB plasma. Control (white circles) had no added erythropoietin; stimulated (black circles) had 0.1 unit per ml. Each point is the mean of triplicates and indicates the end of a 6 hr incubation period with 3 μCi ^{59}Fe per dish. The heme synthesis rate is expressed as a percentage of the value found in the first 6 hr incubation.

to that of erythroblasts surviving since the beginning of culture. If large number of erythroblasts remained in the control culture after 72 hr incubation, the response to EP may be masked. It seems, however, that this is not the case both with AML and with ALL since there is a striking reduction in numbers of erythroblasts prior to culture. Therefore, in the cases of ALL marrow cells seem to have been preserving their capacity to respond to erythropoietin.

These results suggest that there may be a difference between the 2 types of leukemia with respect to the mode of involvement of erythroid cells. It may be hypothesized that there is a more specific suppressive mechanism of erythroid cells in the case of AML than ALL. For example, the number of ERC's and their progenies may have been decreased more intensively in AML or their responsiveness to EP may be lowered.

It is also reported that the urinary excretion of EP (2) or colony-stimulating factor is intact in ALL but suppressed in AML (8). It remains to be elucidated if these observations are relevant to the present studies.

Above hypotheses need a more advanced and detailed study with more cases of the disease. Studies using newer techniques in the formation of erythroid as well as granuloid colonies are in progress.

Summary. In vitro response to erythropoietin of the bone marrow cells from patients with AML and ALL was studied. In the cases of AML the response was apparently suppressed, whereas in cases of ALL the response was well preserved although there was a marked reduction in numbers of erythroblast in the bone marrow of these patients before culture.

These results suggest that there may be a difference between the two types of leukemia with respect to the mode of involvement of the erythron.

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