Stimulation of Alpha and Beta Polypeptide Chain Synthesis in Cultured Human Marrow by Erythropoietin[1] (36768)

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Erythropoietin (ESF) acts on erythroid progenitor cells inducing their differentiation into definitive erythroblasts (1, 2). Associated with this ESF effect there is, in many species, an increase in the rate of heme synthesis (2–5). In addition, in lower mammals, both *in vivo* and *in vitro*, the hormone has been shown to increase the rate of globin synthesis (6). There is also evidence that ESF stimulates globin formation in cultured human marrow cells (7, 8).

Further studies on the mechanism of action of ESF have shown that in cultured rodent marrow cells the increase of hemoglobin synthesis by ESF is preceded by an increase in the formation of 9S RNA which may represent globin polypeptide messenger (9). Indirect support for these results has been the increase in production of alpha, beta and gamma globin polypeptide chains by ESF in cultured neonatal calf marrow cells (10).

The present studies were undertaken to define further the mechanism of action of ESF on human marrow cells growing in primary culture. The data obtained indicate that associated with the increase in the rate of heme and globin synthesis (8) ESF causes a comparable increase in the rate of synthesis of both the alpha and beta polypeptide chains of globin in these cultured marrow cells.

Materials and Method. Culture of bone marrow cells. Human bone marrow cells were obtained by sternal aspiration in patients undergoing this procedure as part of an evaluation for possible metastatic pulmonary carcinoma. Only morphologically normal aspirates were used. The aspirated marrow cells were immediately placed in cold NCTC 109[2] solution and cultured by the method of Krantz (4). In brief, bone marrow cells were washed twice with cold NCTC 109 solution and then suspended in an incubation medium consisting of 60% NCTC 109 solution, 40% heat-inactivated human plasma of AB type and 50 units/ml of penicillin so as to obtain a cell concentration of 2000 nucleated cells (approx 25% erythroid)/mm^3 of medium.

Aliquots (0.8 ml) of this cell suspension were placed into 35 × 10 mm plastic petri dishes[3] and Step III ESF[4] added in a volume of 2 μl of vehicle to give a final concentration of 0.2 units/ml medium. The ESF preparation was dissolved in saline and sterilized by passage through a Millipore filter prior to use. Control cultures were treated with saline alone. Cell cultures were incubated in an atmosphere of 5% CO_2 in air at 37° for 72 hr.

Twenty-four hours prior to the termination of incubation either 1 μCi of uniformly labeled L-valine-^{14}C[5] or 1 μCi of $^{59}FeCl_3$[6] each dissolved in 0.1 ml of type AB plasma was added to each culture dish. At the termination of the incubation period the cells from each culture dish were washed and centrifuged three times with cold phosphate buffered saline (pH 7.4). The cells were then freeze-thawed three times and the hemolysis

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[2] Supplied by Difco Laboratories, Detroit, MI.

[3] Supplied by Falcon Plastics, Los Angeles, CA.

[4] Sheep erythropoietin (200 μ/58 mg) supplied by Connaught Laboratories, Toronto, Canada.

[5] Supplied by International Chemical & Nuclear Corp., Waltham, MA (sp act of 175 mCi/mmole).

[6] Supplied by Mallinckrodt/nuclear, St. Louis, MO (sp act 7.5 mCi/mg).

was completed by the addition of 1 ml of cold distilled water.

Extraction of heme and determination of its radioactivity. To each hemolysate, as prepared above, was added 1 ml of twice concentrated Drabkin's solution. Following this, hemolysates were kept at 4° for 12 hr and then centrifuged at 10,000*g* for 20 min to remove the stroma. Heme was then extracted by the method of Teale (11) into acid methyl ethyl ketone. The methyl ethyl ketone extracts from each tube were removed *in toto* evaporated to dryness on steel planchets, and the radioactivity measured at infinite thinness in a gas-flow counter (background, 22 cpm).

Isolation of hemoglobin and determination of its specific radioactivity. The rate of globin synthesis was determined by measuring the incorporation of ^{14}C-valine into hemoglobin isolated by column chromatography using a modification of the method of Chernoff, Pettit and Northrup (12). For these experiments marrow cells cultured with ^{14}C-valine were washed three times in phosphate buffered saline (pH 7.4), then hemolyzed by freeze-thawing twice and followed by addition of 1 ml of distilled water. Lipids were removed by the addition of 1 ml of carbon tetrachloride and subsequent centrifugation. The resultant hemolysate was dialyzed against potassium phosphate buffer (0.01 *M*, pH 6.2) for 24 hr at 4°. This dialyzed hemolysate was applied to a carboxymethyl cellulose[7] column measuring 1 × 5 cm equilibrated with the dialysate buffer. The hemoglobin was eluted from the column with a two step potassium phosphate buffer (0.01 *M*, pH 6.2–8.2) and quantitated in the eluate by spectrophotometry (8). A 1 ml aliquot of the eluted hemoglobin was pipetted into a liquid scintillation counting vial[8] containing 2 drops of 30% H_2O_2 and 15 ml of counting solution (4.9 g/liter PPO[9], 0.1 g/liter POPOP[9] 2 vol toluene: 1 vol Triton-X[10]). Radioactivity was determined in a Beckman Model LS-250 liquid scintillation counter. Automatic external standard counting did not reveal variable quenching in the samples. The counting error was less than 5%. Specific activity was computed as counts per minute per absorbance units (cpm/OD).

Isolation of alpha and beta polypeptide chains and determination of their specific radioactivities. Since the total amount of hemoglobin in each culture dish was only approximately 3 mg additional nonradioactive hemoglobin was added to each culture. This carrier hemoglobin was obtained from a normal volunteer and 60 mg in the form of a red cell hemolysate was added to each dish. To this supplemented hemolysate (approx 2 ml) was added 0.4 ml of CCl_4. Following centrifugation at 10,000*g* for 20 min, the hemoglobin layer was removed and mixed with 20 vol of 2% acid acetone at −20° to precipitate the globin. The globin was then washed 3 times in acetone at 20° dried under N_2, and dissolved in distilled water and lyophilized.

From this globin preparation alpha and beta chains were isolated by a modification of the method of Clegg, Naughton, and Weatherall (13). Approximately 30 mg of the lyophilized globin was dissolved in 3 ml of a buffer containing 8 *M* urea; 0.005 *M* Na_2HPO_4 and 0.046 *M* 2-mercaptoethanol with the pH adjusted to 6.7 with 85% phosphoric acid. Prior to use the urea was deionized by passing through a column of Rexin I-300.[11] This globin solution was dialyzed against a similarly constituted buffer for 2.5 hr and the dialysis buffer was changed 3 times during this period.

The dialyzed globin was then applied to a carboxymethyl cellulose column (1 × 10 cm) which was previously equilibrated with the urea buffer. The globin polypeptide chains were eluted from the column with a linear gradient system delivered from a gradient mixing apparatus. The first chamber contained 100 ml of the starting urea buffer and the second chamber contained 100 ml of a similar buffer except that the Na_2HPO_4 was at a concentration of 0.03 *M*. Flow rate was regulated at 2 ml/min with a Buchner pol-

[7] Whatman CM23, supplied by W & R Balston Ltd., England.

[8] Supplied by Tracerlab, Waltham, MA.

[9] Supplied by International Chemical & Nuclear Corp., Waltham, MA.

[10] Supplied by Eastman Organic Chemicals, Rochester, NY.

[11] Supplied by Fisher Scientific Co., Fairlawn, NJ.

ystatic pump and 3 ml fractions were collected. The optical density at 280 nm of each of the collected fractions was determined in a Beckman DU-2 spectrophotometer.

Two milliliter aliquots of each of the above fractions were added to 15 ml of counting solution constituted as described in the section on hemoglobin isolation except for the elimination of the H_2O_2. The radioactivity of these aliquots was determined in a Beckman liquid scintillation counter. Specific activity was computed as counts per minute per absorbance units (cpm/OD). The calculation was performed only on homogeneous fractions which immediately surrounded and included the maximum optical densities of the separated chains. The specific activity of each fraction from the same peak agreed with one another within 5%. The rates of synthesis of the alpha and beta chains were compared after correction for the difference in 280 nm absorption and valine number of the two chains.[12]

Results. Figure 1 depicts typical chromatographic separations of globin derived from both a control and an ESF treated culture into alpha and beta chains. The identity of these alpha and beta chain peaks have been confirmed by Clegg, Naughton and Weatherall (13). A small peak emerged just prior to the beta chain peak (Fig. 1) as noted by other investigators (13, 14). For uniformity in calculation, only the tubes at the peak and around the descending border were used for the calculation of beta chain specific activity. Specific absorption for alpha chains at 280 nm is decreased relative to beta chains [as previously noted by others (13, 14)] and is probably due to the difference in the number of tryptophan residues in each chain.

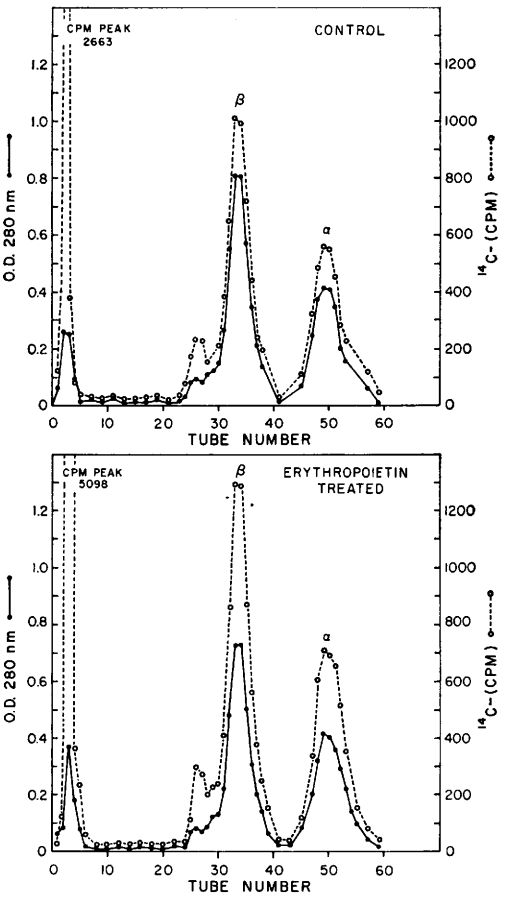

FIG. 1. Typical ionic gradient separation on a carboxymethyl cellulose column at pH 6.7 of alpha and beta chains from the hemolysates prepared from control and erythropoietin treated marrow cultures. (Absorbancy is uncorrected.) High specific activity early peak (tubes 1–5) represents nonglobin protein and free ^{14}C-labeled valine.

Table I summarizes the results of the experiments on synthesis of heme, globin and globin polypeptide chains. In both Expts. 1 and 2, there was significant ($p < .01$) stimulation by ESF of heme, globin, alpha chain and beta chain synthesis. ESF caused a 160% (Expt. 1) and 140% (Expt. 2) increase in synthesis of these compounds as compared with control values. No difference was noted, in either group of experiments, in the relative degree of stimulation of heme, globin, alpha

[12] The absorptions at 280 nm of isolated alpha and beta chains were compared by dissolving each chain in buffer derived from that fraction of the ionic strength gradient in which the respective chain emerged from the column. The concentration of polypeptide chain in each of these samples was determined by measuring their total nitrogen content and knowing the total number of nitrogen atoms present in each chain. The absorption per milligram of beta chains was found to exceed that of alpha chains by a value of 1.50. This value is almost identical to that obtained by Kan, Schwarz and Nathan (14).

TABLE I. Effect of Erythropoietin on Heme, Globin and Alpha and Beta Chain Synthesis in Cultured Human Marrow Cells.

	Heme[a] (cpm/total heme)	Globin[a] (sp act)[b]	α-Chain[c] (sp act)[d]	β-Chain[c] (sp act)
Expt. 1				
Control	1137.3 ± 44.8	253 ± 5	1279.1 ± 55.2	1289.8 ± 47.3
Erythropoietin[e]	1814.7 ± 17.0[f]	385 ± 9[f]	1941.2 ± 90.1[f]	1927.4 ± 64.2[f]
Expt. 2				
Control	545.5 ± 5.5	56.1 ± 7.4	260.0 ± 4.5	256.6 ± 9.8
Erythropoietin[e]	821.0 ± 29.0[f]	99.7 ± 3.4[f]	340.6 ± 9.4[f]	340.4 ± 10.3[f]

[a] Each value represents the mean ± SE of three cultures.
[b] Sp act = specific activity (cpm/OD).
[c] Each value represents the mean ± SE of six cultures.
[d] Corrected to compare with that of β-chain as described in text.
[e] 0.2 units/ml medium.
[f] Difference from control value significant at $p < .01$.

or beta chains. The relative degree of stimulation of alpha and beta chains by ESF was similar in each experiment.

Discussion. It has been postulated (5) that a part of the ESF effect on erythroid differentiation is the induction of the synthesis of Δ-aminolevulinic acid synthetase. This enzyme limits both heme and globin synthesis in erythroid presursors cells (15) and induction of its synthesis is associated with an increase in the rate of heme formation. This increase in heme formation in erythroid cells is associated with an increased production of globin (15) and globin polypeptide chain synthesis (16).

The demonstration by several investigators that ESF stimulates both heme (3–6) and globin (6–8) synthesis in various erythroid systems supports the possibility that one of the primary effects of ESF is the induction of Δ-aminolevulinic acid synthetase. Also, it has recently been shown that ESF stimulates the synthesis of the alpha, beta and gamma globin polypeptide chains of cultured neonatal calf marrow cells (10). The present study demonstrates that in cultured human marrow cells the action of ESF is associated not only with enhancement of heme and globin production (8), but also with a comparable degree of stimulation of both alpha and beta chain synthesis. The data (Table I) indicate that ESF causes no significant difference in the relative rates of synthesis of alpha and beta polypeptide chains. However, since in these experiments marrow cells were incubated with radioactive amino acid for a period of 24 hr the possibility of an early primary preferential ESF effect on one polypeptide chain can not be eliminated by these studies. The finding of an equal degree of stimulation of both heme and globin polypeptide chain synthesis by ESF also lends further support to previous work (17) demonstrating coordinate synthesis of these two compounds.

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