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EVALUATION OF COMMERCIAL ERYTHROPOIETIN ACTIVITY AFTER PREPARATIVE ISOELECTRIC FOCUSING

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SUMMARY: The in vitro study of erythropoiesis is often hampered by impurities associated with commerical preparations of erythropoietin. This report describes the activities associated with commercial erythropoietin, as well as a rapid and convenient method, preparative isoelectric focusing, to enhance the purity of low specific activity erythropoietin preparations.

INTRODUCTION

Erythropoietin, a glycoprotein, is believed to be the principle humoral agent regulating red cell production (1). In vitro erythropoiesis in fact appears to have an absolute requirement for erythropoietin to stimulate or promote the maturation of the developing red cell precursor to a hemoglobin synthesizing stage (2). Unfortunately, commercially available erythropoietin is generally of low specific activity, which complicates the in vitro study of erythropoiesis. This manuscript reports the isolation and analysis of a number of active materials associated with commercial erythropoietin.

METHODS

Isoelectric focusing was performed with an LKB Multiphor unit. Preparative gels were cast in Sephadex G-75 Superfine. Ampholines were selected to generate a 3.5 to 9.5 pH range (0.3% pH 4.6; 0.3% pH 5-7; 4.4% pH 3-10). Samples were applied to established gel geds, and were not included in the original slurry when the gel was poured. After overnight electrofocusing (14-16 hrs.), the gel surface was blotted, and the blot stained with Coomasie brilliant blue to identify the protein bands. The gel surface was cut into 30 equal sections, and eluted with 1.1 ml of water to recover the erythropoietic activity. Eluates from some runs were dialyzed to remove ampholines, but this had no discernible effect on the activity of the fractions, which were assayed for activity both before and after dialysis. Erythropoietin (Step III) was purchased from Connaught Laboratories.

Activity of eluted samples was routinely determined in the fetal mouse liver erythroid system, following either one of two procedures (3,5). In general, livers

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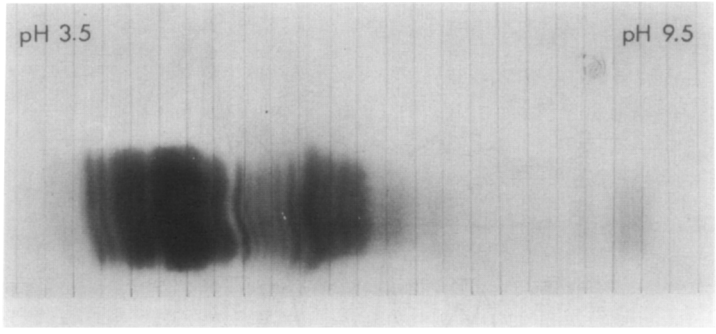

Figure 1. Preparative isoelectric focusing of erythropoietin. After commercial erythropoietin (10 units) was electrofocused (pH 3.5 to 9.5). The Sephadex bed was blotted and the paper blot stained with Coomasie brilliant blue. Light regular lines are pencilled marks which indicate lines along which gel was sliced for assay. The acidic end of the gel is to the left.

were obtained from embryos of C3H mice at 14 days of gestation. Cells were disaggregated by repeated gentle aspiration through a Pasteur pipette, and then suspended in α-Media (Gibco) and 5% fetal calf serum (Gibco). Cells were cultured at 37° and 5% CO_2 for 16 hours in the presence of test materials and pulsed for a final 4 hours with ^{3}H-leucine (New England Nuclear) to assay effects upon protein synthesis. Cell suspensions contained 0.5×10^{6} cells/ml and were established in triplicate.

Reactions were terminated by adding an excess of cold saline to the culture tubes. After the cells were pelleted by centrifugation, they were resuspended and swollen in 10 ml of water. Total protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA). After the discharge of amino acyl transfer RNA complexes by heating at 90° for 20 min., precipitates collected on millipore filters were transferred to scintillation vials and, after the addition of formic acid and scintillation cocktail (Scintiverse, Fisher Scientific), radioactivity was determined in a Packard automatic scintillation spectrometer.

Effects upon proliferation were monitored by four hour pulse with ^{125} I-UdR (Amersham) after overnight culture. In this assay the procedure of Napier et al. using microtiter plates and automatic cell harvesting was followed exactly (5).

RESULTS

After electrofocusing for 16 hours more than 25 protein bands could be detected by Coomasie brilliant blue staining (fig. 1). In this particular run 3.0 milligrams of Connaught Step III erythropoietin were electrofocused. As many as 40 milligrams have been applied to other runs without significant loss of resolution. The light regular vertical lines in figure 1 are pencilled marks which indicate the lines along which the sections were cut and eluted.

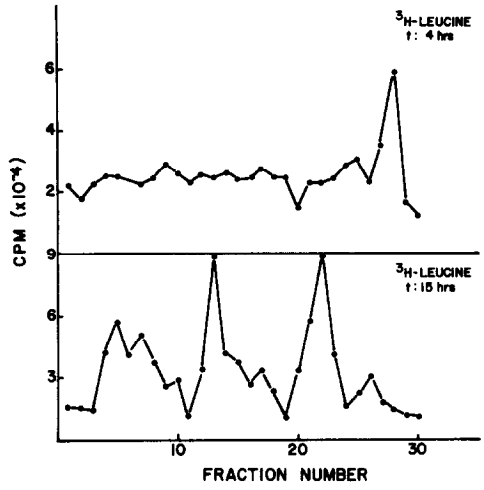

Figure 2. Effect upon ^{3}H-leucine incorporation of erythropoietin (Ep) preparations fractionated by preparative isoelectric focusing. Ten units of Ep were found on a pH gradient 3.5 to 9.5. Each of 30 fractions was eluted with 1.1 ml of water and 25 µl of each was assayed in the fetal mouse system. In the top panel cells were exposed to test material for 4 hrs.; in the bottom panel cells were exposed for 15 hrs. before a final 4 hr. pulse with ^{3}H-leucine. All assays were performed in triplicate.

After elution the samples were assayed for their ability to influence leucine incorporation after different times of exposure (fig. 2). When cells were exposed to the fractionated Ep for 4 hours, increased incorporation was observed only in the assay tubes containing fractions 27 and 28. In contrast, when cells were exposed to the fractions for 15 hours prior to the final pulse for 4 hours with ^{3}H-leucine multiple regions of activity were detected. Major areas of activity after 15 hours were identified in the ranges 3.8 -4.2 (fxns. 4 - 7), pH 5.5 - 5.8 (fxns. 12 - 14), and pH 6.2 - 6.8 (fxns. 21 - 23). It was not surprising that many active regions were detected since sialated as well as asialo-erythropoietins are reported to be active in *in vitro* assay systems. That this may be a reasonable interpretation was reinforced by our observations that sialic acid residues were detected in the first region but not in subsequent ones, and neuraminidase treatment prior to electrofocusing removed the first region and reinforced the activity observed in the second region. In these particular experiments, the results of which are shown in figure 2, the eluted fractions were not dialyzed to remove ampholines prior to assay. However, no

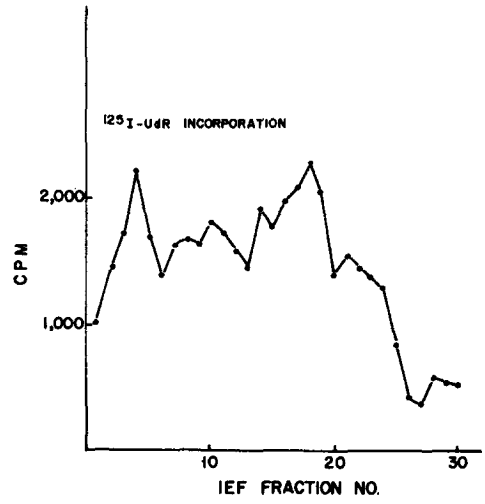

Figure 3. Effect upon ^{125}I-UdR incorporation of erythropoietin (Ep) preparations fractionated by preparative isoelectric focusing. Cells were exposed to test materials for 15 hrs. gefore a final 4 hr. pulse with ^{125}I-UdR. All assays were performed in triplicate.

differences have been reported in activity in any preparations assayed before and after dialysis. It should also be noted that no change in electrofocusing pattern was observed when erythropoietin preparations were dialyzed for 5 hours at pH ranging from 3.5 to 8.0. This was done to insure that the sialic acid residues were not excessively pH labile, and thus potentially lost during the electrofocusing.

Ability of the fractions to stimulate proliferation was assayed by testing their effect upon ^{125}I-UdR incorporation (fig. 3). The three regions of activity observed in the 15 hr assay of ^{3}H-leucine incorporation were again noted, but the peak of activity in the region of fraction 27 was absent. Neither in the run shown, nor in any subsequent has a capacity for stimulation of proliferation been observed in this last region which suggests that the stimulation of leucine incorporation is attributed most likely to an effect at the level of transcription.

Based on observations that the addition of stock erythropoietin to active fractions either reduced incorporation or had only a slight positive influence (fig. 4). The conclusion was reached that preparative isoelectric focusing not only enhanced the purity of erythropoietin but also enhanced activity by removing possible inhibitory substances.

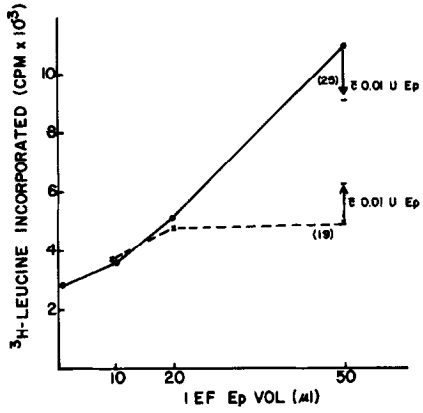

Figure 4. Effect of varying concentrations of selected fractions of erythropoietin separated by isoelectric focusing. Material from fractions 19 and 25 were added to 14 day fetal mouse liver erythroid cells cultured for 18 hours. ^{3}H-leucine was added for the final four hours of the reaction. Vertical arrows at highest concentrations indicate effect upon incorporation of added commercial erythropoietin (0.10 U). All cultures were established in triplicate.

DISCUSSION

The results of this study suggest that four major regions of biologically active material can be separated by isoelectric focusing from commercial preparations of erythropoietin. Consistent with previous reports that the native erythropoietin molecule contains sialic acid (6) and has an isoelectric point of approximately 4, (4), the first active region (fractions 4 - 7) has been found to contain sialic acid, and has an apparent isoelectric point of 4.1. The second major region (fractions 12 - 14) apparently represents the asialo erythropoietin molecule based on experiments in which the early region was displaced by treatment with neuraminidase and activity in the second region was enhanced (data not shown). The fourth fraction (fractions 27 - 28) is apparently unrelated to any of the first three, since it alone stimulates leucine incorporation within four hours. The relationship, if any, of the third region to any other area of activity has not as yet been determined. In general, commercial erythropoietin preparations contain three major fractions which exert stimulatory influences on proliferation, and one fraction which apparently stimulates transcription.

Specific activity of the separated fractions was not determined at this time because of the multiple agents identified. In particular, both sialated and asialo erythropoietin are active in vitro whereas only the sialated form is reported to be active in vivo. In addition, in our tests stock commercial erythropoietin generally plateaued in activity before 0.2 unit/ml at a 2-fold stimulation. Active fractions after isoelectric focusing, on the other hand, routinely showed no deviation from a linear dose response at levels of stimulation which exceeded five fold. The increased levels of stimulation possible after isoelectric focusing are consistent not only with enrichment of the preparation for the erythropoietin molecule, but also with removal of some inhibitory influences.

In summary, preparative isoelectric focusing appears to be a convenient and efficient procedure by which to enhance the activity of commercial erythropoietin preparations, thus facilitating in vitro studies of erythropoiesis. Furthermore, studies indicate that by this procedure different active materials may be isolated which may have diverse effects upon various stages of erythropoiesis, e.g. proliferation and transcription.

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REFERENCES

1. Miyake, T., Kung, C.K.-H. and Goldwasser, E. (1977) J. Biol. Chem. 252, 5558-5564.
2. Eaves, C.J., Humphries, R.K. and Eaves, A.C. (1979) *In* Cellular and Molecular Regulation of Hemoglobin Switching. Stammatoyannopoulos, G. and Nienhuis, A. edd. pp. 251-273, Grune & Stratton, New York.
3. Fuhr, J.E. and Dunn, C.D.R. (1979) Exp. Hemat. 7, 490-494.
4. Puschmann, M., Thorn, W. and Yen, Y. (1978) Res. Exp. Med. 173, 293-296.
5. Napier, J.A.F. and Evans, J. (1980) Clin. Lab. Haemat. 2, 13-19.
6. Lukowsky, W.A. and Painter, R.H. (1972) Can. J. Biochem. 50, 909-917.