Incomplete Erythropoietin Activity in Normal Plasma Components[1] (35714)

PETER P. DUKES AND DENMAN HAMMOND
With the technical assistance of C. Greene, J. Price and C. Polk
Division of Hematology, Childrens Hospital of Los Angeles, and Departments of Biochemistry and Pediatrics, University of Southern California School of Medicine, Los Angeles, California 90027

Erythropoietin, the humoral regulator of red cell production which is elicited in response to tissue hypoxia, has been demonstrated in the body fluids of humans and animals. We have recently reported that erythropoietin preparations behave as complexes of different activities which may be detected *in vivo* and/or *in vitro* (1). Certain erythropoietin preparations exhibited much higher activities *in vitro* than *in vivo* when they were related to a common standard. This suggested that they contained factors which only influenced the rate of certain partial processes of erythropoiesis such as those measured by increased glucosamine incorporation or heme synthesis. Erythropoietin preparations treated by neuraminidase (EC 3.2.1.18) have been reported by Goldwasser and co-workers (2) to have lost *in vivo* but not *in vitro* activities. A plasma precursor which may be transformed into erythropoietin active *in vivo* has been reported on by Kuratowska (3) and by Gordon and coworkers (4). Clinical studies from our laboratory (5) showed that it is possible to initiate a wave of erythropoiesis in the marrow of patients with hypoplastic anemia by fresh plasma infusions. A component of normal plasma seems to be required by these patients although they exhibit high circulatory levels of erythropoietin as measured by the *in vivo* assay in mice. All these findings made it seem possible that in normal plasma, there exist factors which, although not normally rate limiting, are involved in the regulation of erythropoiesis in a nontrivial (nonnutritional) fashion. In this communication, we present evidence for the existence of factors in Cohn fraction III of normal plasma which behave like incomplete erythropoietin since they stimulate glucosamine incorporation and heme synthesis in cultured rat marrow cells but are inactive *in vivo*.

Materials and Methods. We are greatly indebted to Dr. E. Shanbrom, Hyland Division, Travenol Laboratories, for putting at our disposal a complete set of human plasma Cohn fractions (6, 7) and prothrombin complex. Other plasma fractions were purchased from commercial sources. We are grateful to the National Heart and Lung Institute for supplying us with human urinary erythropoietin which had been collected by the Department of Physiology, University of the Northeast, Corrientes, Argentina, and was processed further by our laboratory.

Erythropoietin assays were performed as described previously (1). Briefly, the *in vivo* assay was based on measuring ^{59}Fe incorporation into newly formed red cells of mice made polycythemic by hypoxia (8). For the *in vitro* assays, rat bone marrow cells were incubated (9) in a medium of 47.5% NCTC 135, 47.5% calf serum, and 5% iron-saturated human serum at 37° in a humidified atmosphere of 95% air, 5% CO_2. The 0-18-hr incorporation of ^{14}C-glucosamine into cellular constituents or the 42- to 46-hr incorporation of ^{59}Fe into heme extracted from the cells was measured. Response (Y) was expressed as percentage of increase over controls. Log response (log Y) was plotted against log dose (log X), the slope (b) of the linearized portion of the dose–response curve was calcu-

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lated by least-square best-fit approximation of the regression of Y on X as the parabolic function $Y = aX^b$. In all assays, the same erythropoietin laboratory standard, an anemic human urine extract (UE-1), which had been standardized in terms of standard A, was employed.

Results and Discussion. The results of three kinds of erythropoietin assays of Cohn fractions of normal, nonanemic human plasma are given in Table I. Significant activity in the ^{14}C-glucosamine and ^{59}Fe-heme systems which differed in magnitude in the two systems was found in fraction III. As one might expect, the immediate precursors of fraction III, namely, II + III W and II + III also showed lesser amounts of these activities. The prothrombin complex (clotting factors II, VII, IX, X) listed in Table I also exhibited some activity. It had been prepared from Cohn fraction III by adsorption to tricalcium phosphate (10).

All the plasma fractions had to be assayed as partial suspensions since they were not fully soluble at the concentrations used: 2–6 mg/mouse in 0.4 ml physiological saline and 62.5–250 μg/ml tissue culture medium, respectively.

Fractions III from other human sources and from cow and rabbit plasma also showed activity in the glucosamine assay system (Table II). These fractions differed from lot to lot in potency but all contained measurable activity, demonstrating that such activity from normal plasma is concentrated in fraction III by the Cohn procedure.

A comparison of the log dose–log response slopes (b) of active plasma fractions and of an erythropoietin standard is presented in Table III. In both *in vitro* assay systems, the b value of the Cohn plasma fraction II + III, III, and the water-extracted residue of III was virtually identical with the b value of the erythropoietin standard obtained with aliquots of the same cell culture, which means that dose–response regression lines (within certain dose limits) paralleled each other.

Fraction III was serially extracted with water (3 × 200 ml/g) and 1 M ammonium

TABLE I. Survey of Cohn Fractions of Normal Human Plasma for Erythropoietin Activity.

Preparation (characteristic component)	Specific activity (u/mg)		
	In vivo (mouse)	In vitro (^{14}C-glucosamine)	In vitro (^{59}Fe-heme)
I (fibrinogen)	0	0	0
II + III	Trace < 0.007	0.14 ± 0.04 (9)[a]	Trace
III-0 (lipoprotein)	Not tested	0	Not tested
II + III-W	0	0.15 (0.05, 0.25)[b]	Trace
III (β-globulin)	Trace < 0.007	1.96 ± 0.11 (8)[a]	4.87 ± 0.51 (3)[a]
III-1 (isoagglutinin)	0	Trace	0
Prothrombin complex	Not tested	0.06	Not tested
II (γ-globulin)	0	0	0
IV-1 + IV-4	0	Not tested	Not tested
IV-1	0	0	Trace
IV-4 (transferrin)	0	0	0
IV-5	0	Not tested	Not tested
IV-6	0	Not tested	Not tested
IV-8	0	Not tested	Not tested
IV-9	0	Not tested	Not tested
IV-5 + IV-6	0	Not tested	Not tested
V (albumin)	0	0	0
VI	Not tested	0	0

[a] Mean ± standard error; number of assay points in quadruplicate used in calculation of mean in parentheses.

[b] Mean; assay points used in calculation of mean in parentheses.

TABLE II. Comparison of the Activity of Cohn Fractions III in the ^{14}C-Glucosamine Incorporation Assay.

Source of fraction III	Stimulation (% increase over control ± SEM)[a]	Specific activity (u/mg)
Hyland Laboratories (human)		1.96[b]
Nutritional Biochemicals Corp. (human lot No. 8192)	9.5 ± 1.9	0.30
Pentex Biochemicals (human lot No. 16)	45.1 ± 0.7	3.34
Pentex Biochemicals (human lot No. 17)	7.8 ± 2.7	0.22
Mann Research Laboratories (human lot No. 04113)	7.2 ± 1.5	0.19
Pentex Biochemicals (bovine lot No. 12)	12.0 ± 4.6	0.43
Pentex Biochemicals (rabbit lot No. 10)	39.0 ± 2.8	2.70

[a] All specific activity estimates with the exception of the first preparation are based on means of an assay done in quadruplicate at the 62.5 μg/ml level.

[b] Based on eight means (assay points at different dose levels; see Table I).

acetate (8 × 200 ml/g) at 0–4° in order to solubilize and concentrate the observed activities. The water extracts did not contain any activity.

For the residue, measured by the ^{14}C-glucosamine assay, recovery was 72%, specific activity was 1.9 u/mg (0.97 of the starting material, see Table I) whereas when measured by the ^{59}Fe-heme assay, recovery was only 21% and specific activity had decreased to 1.35 u/mg (0.28 of the starting material, see Table I).

One-molar ammonium acetate extraction of the water residue, followed by lyophilization of the extracts to remove the volatile salt, yielded a minute amount of solubilized activity as measured by both systems without enrichment. The ammonium acetate residue

TABLE III. Comparison of Log Dose–Log Response Slopes of Normal Plasma Fractions and of a Standard Urinary Erythropoietin in Two *in Vitro* Assays.

Description of plasma fraction	^{14}C-Glucosamine assay				^{59}Fe-Heme assay			
	Test preparation		Erythropoietin standard[a]		Test preparation		Erythropoietin standard	
	b[b] ± s_b	n	b ± s_b	n	b ± s_b	n	b ± s_b	n
Normal human, Cohn II + III	0.49 ± 0.06	12	0.47 ± 0.06	12	—		—	
Normal human, Cohn III	0.69 ± 0.04	16	0.75 ± 0.03	12	0.30 ± 0.10	12	0.29 ± 0.17	11
Residue of water extraction of normal human, Cohn III	0.65 ± 0.05	12	0.56 ± 0.04	10	0.37 ± 0.25	11	0.63 ± 0.38	10

[a] Anemic human urine extract (UE-1).

[b] Dose–response slope (b) ± standard deviation of dose–response slope (s_b); number of data points used in constructing regression (n).

contained all of the input glucosamine-type activity with the specific activity improved to 3.37 u/mg, while at the same time it had lost 99% of its ^{59}Fe-heme-type activity.

The observed parallelism of dose–response lines of test and erythropoietin standard preparations favors the possible identity or close similarity of the factors in normal plasma and standard erythropoietin which cause increased glucosamine uptake or heme synthesis, respectively. On the other hand, the dissimilarity of specific activities (Table I) and the different recovery and stability obtained by extraction procedures, as recorded by the two *in vitro* assay systems, suggest that more than one factor is involved in the stimulation of the *in vitro* assay systems. These features strengthen our hypothesis (1) that erythropoietin is a complex of several different biologically active entities.

Summary. Cohn fractions of normal human plasma were surveyed for erythropoietin activity by an *in vivo* and two *in vitro* assay systems. Fractions II + III, II + III W, and especially fraction III, were found to stimulate glucosamine incorporation and heme synthesis of marrow cells in culture. Log dose–log response regression lines of plasma fractions and of an erythropoietin standard were found to be parallel. Only traces of activity could be detected by the exhypoxic polycythemic mouse assay. Fraction III from several different sources and species was found to be active *in vitro*. A human fraction III was shown to have a different specific activity relative to a common erythropoietin standard in the two *in vitro* assays. Subfractionation of fraction III by extraction procedures demonstrated low stability for the activity measured by the ^{59}Fe-heme assay, whereas it was possible to obtain without loss a preparation enriched in the activity stimulating glucosamine incorporation.

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