Erythropoietic Activity of Untreated and Deproteinized Normal Human Plasma.* (29982)

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While the presence of erythropoietin in plasma derived from a variety of experimental and clinical anemias(1,2) has been repeatedly confirmed, its existence in normal plasma remains to be proved. Several workers have reported demonstrable erythropoietic activity in unconcentrated normal plasma from human(3,4) and animal(5,6) sources, but such activity usually has been of borderline significance. Heat denatured fractions are stated to be active in 10-fold concentrations but not effective when unconcentrated (7). Others have been unable to detect activity in extracts of concentrated normal plasma(8,9). It has been claimed that the stimulatory effects of intact plasma observed by some may not be due to erythropoietin but may rather reflect an enhancement of erythropoiesis elicited by nutrients in whole plasma when tested in hormonally or nutritionally deprived assay animals(3). It may be that some of the conflicting evidence is attributable to the relatively crude assay methods employed and/or to the use of numbers of animals inadequate for the resolution of small differences. It thus seemed worthwhile to reinvestigate this problem by comparing assays of whole untreated and deproteinized plasma in the transfused polycythemic mouse with results obtained with the less sensitive starved or hypophysectomized rat. The polycythemic mouse, in addition to its increased sensitivity, is also suitable because it is little affected by possible hormonal or nutritional components of intact plasma. In view of its ready availability, human plasma was selected for study.

Methods. Blood was collected from normal (screened for normal hematocrits: 41-46%) male and female volunteers by venipuncture, centrifuged immediately and the plasma stored at —20°C for no longer than 3 weeks before use. Whole plasma samples were not concentrated before injection. Deproteinization of plasma samples was done by a slight modification of the method of Borsook *et al*(10). The pH was adjusted to 5.5 with HCl (diluted 1:1 with distilled H_2O) and the plasma was heated in a boiling water bath for 20 minutes. A volume of boiling distilled H_2O, equal to the original plasma volume, was added and the plasma then filtered through a Fisher vacuum filtrator using 41 H filter paper. The precipitate was resuspended with an additional equal volume of boiling distilled H_2O, boiled for 5 minutes, and the remaining supernatant filtered. The filtrates were pooled, the pH readjusted to 7.0 with 0.1 N NaOH, and concentrated to one-third original volume in a flash evaporator. Samples were frozen until just before use.

Young adult Sprague-Dawley rats, aged 2-3 months (350-400 g) were used for the starved and hypophysectomized rat assays. Animals for the latter were hypophysectomized for at least 40 days prior to use to insure a stable post-operative state. The starved rat assay was carried out by a modification of the procedure of Fried *et al*(11). Food was removed on day 1, but water was permitted for the entire course of the assay. Test animals were given subcutaneous injections of intact plasma or deproteinized extract (DPPE), 1 ml/day/4 days (days 1-4; total dose: 4 ml), while saline-injected or intact animals served as controls; one hour after the last subcutaneous injection 1 μc Fe^{59} citrate was administered i.v.; 24 hours later (day 5) blood was sampled *via* aortic puncture. Hypophysectomized rat assays were carried out in the same manner, except that these animals were permitted both food and water. Hematocrits were taken on all

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blood samples (microhematocrit method); the radioactivity of 1 ml aliquots was determined in a well-type scintillation counter and compared with a previously prepared Fe^{59} standard. Animals with hematocrits less than 40 were discarded. Altogether, 360 rats were used in these assays.

Polycythemic mouse assays were done by a modification of the technique of Filmanowicz and Gurney(12). Swiss Ha/ICR mice, 5 weeks old (30-35 g), were transfused i.p. with washed, homologous red cells (80% hematocrit): 1 ml/day on days 1 and 2, and 0.5 ml on day 3. Fresh blood for transfusion was drawn from donor mice on each day. Individual mice were checked for adequate polycythemia (hematocrit 75-85) *via* tail vein samples on day 6 and suitable animals were given 1 ml or 2 ml of intact plasma or DPPE subcutaneously. Untreated mice served as controls. On day 8, all mice received i.v. injections of 0.5 μc Fe^{59} in 0.5 ml normal saline; 48 hours later (day 10), blood was drawn from the dorsal aorta and hematocrits and radioactivity determined as described above. Mice with hematocrits of less than 60 were discarded. A total of 424 mice was used for this experiment.

Results were expressed in terms of percentage of radioiron uptake of blood, assuming a blood volume of 5% of body weight for both mice and rats. The mean differences between treated groups and the simultaneous control group ($\overline{X}_i$-$\overline{X}_c$) within a given assay were computed and the resultant values judged for significance by the *t* test for one-sided comparisons between *p* treatment means and a control(13).

Results. The data from 4 representative polycythemic mouse assays are plotted in Fig. 1. Two samples were whole untreated plasmas; 2 were DPPE; all mice received either 1 ml untreated plasma or DPPE. Radioiron uptake in control mice was found to be extremely stable, averaging 0.10-0.12%. While absolute differences in radioiron incorporation between controls and samples judged active are admittedly small, it should be pointed out that samples with significant activity evoked 50 to 100% greater responses. Furthermore, if small differences in isotope uptake are to be ascribed to simple variation

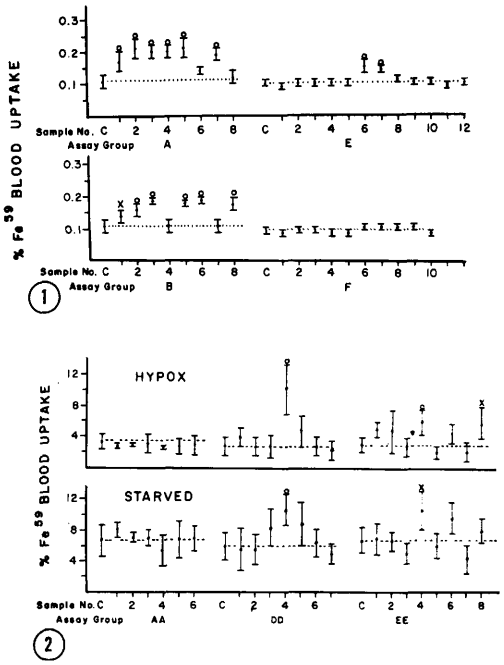

FIG. 1. % Fe^{59} uptake of blood of polycythemic mice given whole untreated (groups A and B) or deproteinized (groups E and F) human plasma. Each point represents the mean ± S.D. of 5 mice. C denotes the control group; a dotted line has been extended from the control value of each assay group to facilitate comparisons. Significant responses with respect to control values are indicated as: ○, $P < 0.01$; ×, $0.05 > P > 0.01$.

FIG. 2. % Fe^{59} uptake of blood of hypophysectomized and starved rats given whole untreated (groups AA, 1-6; EE, 1-4) or deproteinized (groups DD, 1-7; EE, 5-8) human plasma. Each point represents the mean ± S.D. of 3-5 rats. C denotes the control group; a dotted line has been extended from the control value of each assay group to facilitate comparisons. Significant responses with respect to control values are indicated as: ○, $P < 0.01$; ×, $0.05 > P > 0.01$.

in responses, one should expect to see a fairly random fluctuation of responses about the control level: *i.e.*, there should be responses above and below the control value. This was a frequent finding among the deproteinized samples, but in no case did untreated plasma produce a response lower than control level. A summary of the results obtained in all mouse assays using 1 ml untreated plasma or DPPE appears in Table I. Twenty-four out of 37 intact plasma samples were found to be significantly active, whereas only 4 out of 30 DPPE samples were positive. The response of polycythemic mice receiving 2 ml of whole untreated and deproteinized human

TABLE I. Summary of Assays of Whole Untreated and Deproteinized Normal Human Plasma in Polycythemic Mice.*

Group	Plasma treatment	No. samples assayed	No. samples with significant activity†
A	None	8	6
B	"	8	6
C	"	8	7
D	"	13	5
Totals		37	24
E	Deproteinized	13	2
F	"	10	0
G	"	7	2
Totals		30	4

* Each sample assayed in 5 mice, each receiving 1 ml of intact plasma or DPPE subcutaneously.
† Significance judged by "multiple treatment" t test; $P < .01$ as compared to intact controls with the exception of 1 sample in group B, where $.05 > P > .01$.

plasma appears in Table II. Three out of 5 intact plasma samples were found to be significantly active, whereas all the DPPE samples proved negative. Three representative assays of intact and deproteinized plasmas in starved and hypophysectomized rats are plotted in Fig. 2, and a summary of the total results appears in Table III. Of 23 intact plasma samples, only one (EE-4) displayed significant activity in both starved and hypophysectomized animals. One of 11 deproteinized extracts tested (DD-4) was active in both assay animals, while another (EE-8) was active only in hypophysectomized rats. The EE-4 and -8 samples above were active

TABLE II. Summary of Assays of Whole Untreated and Deproteinized Normal Human Plasma in Polycythemic Mice.*

Group	Plasma treatment	Samples with significant activity†
AAA	None	0
	Deproteinized	0
BBB	None	Significant
	Deproteinized	0
CCC	None	Significant
	Deproteinized	0
DDD	None	Significant
	Deproteinized	0
EEE	None	0
	Deproteinized	0

* Each sample assayed in 5-6 mice, each receiving 2 ml of intact plasma or DPPE subcutaneously.
† Significance judged by "multiple treatment" t test; $P < .01$ as compared to intact controls obtained for group BBB, CCC, DDD.

in the transfused mouse, but the DD sample was not tested.

Discussion. Erythropoietic stimulating activity was present in the majority of the whole untreated normal plasma samples assayed in the hypertransfused mouse. It is of interest that 1 ml or 2 ml doses in the mouse proved effective, whereas 4 ml of either intact plasma or DPPE essentially failed to elicit significant responses in the rat assays. Such findings are consistent with the greater sensitivity of the transfused animal to erythropoietin. If, however, the source of the plasma activity is some relatively non-specific factor, at least a small augmentation of the radioiron uptake in whole plasma-treated

TABLE III. Summary of Assays of Whole Untreated and Deproteinized Normal Human Plasma in Starved and Hypophysectomized Rats.

Group	Plasma treatment	No. samples assayed*	No. samples with significant activity†	
			Starved	Hypox
AA	None	6	0	0
BB	"	6	0	0
CC	"	7	0	0
DD	Deprot.	7	1	1
EE	None	4	1	1
	Deprot.	4	0	1

* Each sample assayed in 3-5 test animals.
† Significance judged by "multiple treatment" t test; $P < 0.01$ as compared to intact (AA-CC) or saline-injected (DD-EE) controls.

rats might have been expected. Gurney and Pan(3) reported that 4 ml doses of whole human plasma evoked slight elevations of radioiron incorporation over saline responses in starved rats and marked increases in hypophysectomized rats; boiled extracts proved ineffective in both types of animals. In the studies reported here in rats, the responses evoked by whole plasma, as well as by DPPE, were essentially indistinguishable from those of saline. Such different results obtained in hypophysectomized rats may be related to the use of different strains or to the length of time after hypophysectomy that plasma or extract was tested. Thus, Gurney and Pan employed rats for assay 1-2 weeks post-hypophysectomy. However, similar results to those reported here were found by Stohlman and Brecher(8), who used rats both 8-10 and 30-40 days after pituitary ablation.

One might expect that, if the plasma activity is indeed due to erythropoietin, established dose/response characteristics should be observable. However, 2 ml doses of untreated plasma did not evoke significant responses above those produced by 1 ml. We have also noted only small, insignificant rises in radioiron incorporation with increasing doses of up to 3 ml of human or dog plasma in the polycythemic mouse(14). A possible explanation is that erythropoietin levels of normal plasma are so low that small increases in dose (whether by administration of greater volumes of plasma or by concentration) still reflect the rather flat early portion of the typical dose/response curves obtained for erythropoietin(15). This does not explain, however, the failure of some investigators to elicit positive responses with highly concentrated fractions of normal plasma. The possibility exists that an inhibitor or binding substance present in normal plasma is also being concentrated along with erythropoietin in such preparations.

The reason for lack of activity in some of the intact plasma samples assayed in the transfused mouse remains obscure. It may be that a distribution of levels of erythropoietic activity exists within the blood of the human population. No correlation of the presence or absence of activity with hematocrit levels of donors was evident. Minute losses of activity, even within the short duration of storage, might have been sufficient to preclude positive responses. The reduction in the fraction of positive responses to boiled plasma observed in the transfused mouse is in harmony with results obtained by others (3,8).

The concept that red cell formation is regulated at all times by erythropoietin has been challenged by those who have been unable to demonstrate this hormone in plasma unless significant anemia has occurred. It appears from the present studies that erythropoietic stimulating activity is demonstrable in untreated normal human plasma but that sensitive assay methods and rigorous statistical procedures are required for its detection. Whether this activity actually represents circulating erythropoietin remains to be determined.

Summary. Significant erythropoietic stimulating activity was found in 27 out of 42 samples of untreated normal human plasma assayed in the transfused polycythemic mouse. Deproteinization by boiling of plasma resulted in a marked reduction in the fraction of active samples. Both whole untreated normal plasma and deproteinized extracts were essentially inactive in hypophysectomized and starved rats. The possibility that these results are due to low levels of erythropoietin in normal plasma is discussed.

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