Erythropoietic Activity in Plasma and Urine of Dogs After Bleeding.* (25259)

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Since Reissmann's experiments(1) on parabiotic rats established humoral regulation of erythropoiesis, numerous workers(2-5) demonstrated erythropoietic activity in plasma and urine of several mammalian species made hypoxic or anemic. The only material available for chemical characterization of erythropoietic factors is plasma of rabbits treated with phenylhydrazine or active urine of some anemic patients(6-10). More recently, Hodgson *et al.*(11) proposed that urine of anemic rabbits may serve as a source of erythropoietin. No demonstration has been made of an erythropoietic factor in plasma of dogs after bleeding. The present study demonstrates presence of erythropoietin in plasma and urine of bled dogs.

Material and methods. Six dogs weighing between 12 and 26 kg were bled once or twice daily for 5 to 6 days to hematocrit values from 5 to 11%. Blood volumes were maintained by replacing amount of blood drawn with equal volume of 6% dextran in saline.‡ Plasma samples were frozen and stored until used. Boiled filtrates of normal and anemic plasma were prepared by Borsook's method (12). The material was adjusted to pH 5.5 with 1N HCl, boiled 15 minutes, and filtered. The precipitate was washed with equal volume of distilled water, the wash added to filtrate and samples adjusted to initial volume of plasma. Urine was frozen immediately after collection and assayed without further treatment. Erythropoietic activity of plasma and urine was measured by Fe^{59} red cell incorporation assay(13), using starved female rats of Long-Evans strain weighing 150 to 200 g. After 24 hours starvation each rat received 2 cc plasma or urine subcutaneously daily for 2 successive days. Twenty-four hours after second injection, Fe^{59} (30,000 to 100,000 c/min.; specific activity 2 $\mu c/\gamma$) combined with rat plasma was injected intraperitoneally. Eighteen to 20 hours later, a sample of blood was obtained from aorta and counted. Five to 10 rats were used for each determination. Controls consisted of both uninjected rats and groups receiving normal plasma. Erythropoietic activity of plasma samples from the same dog obtained at different states of anemia was compared to normal plasma obtained by first venesection. Hematocrits were measured in Wintrobe tubes by centrifugation at 3,000 rpm for 30 minutes. Platelet determinations were made by direct technique using Rees-Ecker solution as diluent and leukocytes with 2% acetic acid.

Results. Plasma of bled dogs contains a factor capable of stimulating Fe^{59} red cell uptake in starved rats (Fig. 1). When hematocrit was above 16%, no activity was demonstrated in plasma. Although activity was present in plasma from dogs bled to hematocrit of 12% (dog 4: 11.4% uptake and dog 6: 15.4%), a more striking response was obtained when the hematocrit was 9% or less (dog 2: 30% uptake, dog 4: 30.9%).

Urine samples from 3 dogs were assayed (Fig. 2). Activity was obtained from urine of 2 dogs whose hematocrits were 8% and 9%: 26.9% Fe^{59} uptake after injection of urine from dog 5 and 17.8% after urine injection from dog 6. Urine from a third dog whose hematocrit was 11% was inactive: 4.2% uptake. This urine was also inactive after dialysis for 24 hours at 4°C (4.8% uptake).

The plasma retained activity after remaining at room temperature for 24 hours but plasma and urine activity often decreased after weeks of storage in frozen state (Table

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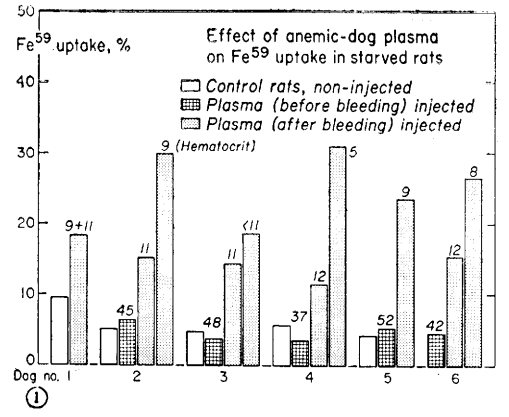

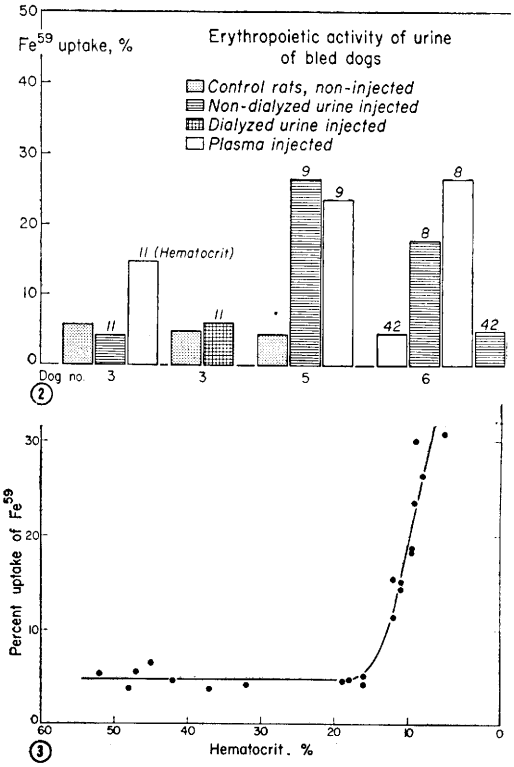

FIG. 1. Hematocrit value at time of withdrawal of plasma is given at top of each column.

FIG. 2. Erythropoietic activity of urine sample compared with correspondent plasma. Figure at top of each column indicates hematocrit value at time when material was collected.

FIG. 3. Values of erythropoietic activity of plasma in regard to hematocrit measurements.

I). Plasma activity was partially destroyed by boiling 15 minutes (Table II).

Injection of active erythropoietic plasma for 2 consecutive days did not alter number of white cells or platelets of recipient rat.

Discussion. When sufficient anemia is produced, erythropoietic activity appears in dog plasma, but the dog is not as responsive to bleeding as rabbit, rat, or monkey. In these species, others(2-4,14) have demonstrated erythropoietic activity when the hematocrit is 20% and hemoglobin is 6 g%. However, assay methods were different from that used in our investigation. It is difficult to demonstrate activity in plasma of dogs because it is necessary to produce severe anemia, which requires frequent venesections and careful maintenance of blood volume with plasma substitute (Dextran) to avoid fatal shock.

TABLE I. Effect of Storage in Freezer (−18°C) on Erythropoietic Activity of Plasma and Urine.

Sample No.	Date of assay	% Fe^{59} uptake	No. recipient rats
Plasma 6.19	12/25/58	26.4 ± 5.2*	9
Controls†	"	4.6 ± 2.4	9
Plasma 6.19	1/ 9/59	21.9 ± 4.6	6
Controls	"	5.6 ± 1.4	6
Plasma 6.23	"	14.8 ± 4.3	6
Controls	"	5.6 ± 1.4	6
Plasma 6.23	1/23/59	8.2 ± 1.5	6
Controls	"	3.1 ± .6	6
Plasma 34.8	12/12/58	30.9 ± 4.9	10
Controls	"	5.7 ± .89	10
Plasma 34.8	2/20/59	15.5 ± 5.6	6
Controls	"	3.3 ± 1	8
Urine 36.19	12/25/58	17.8 ± 4.8	10
Controls†	"	4.6 ± 2.4	9
Urine 36.19	1/30/59	19.7 ± 9.4	7
Controls	"	4.6 ± .79	6
Urine 35.14	12/20/58	26.4 ± 8.2	10
Controls	"	4.3 ± .70	10
Urine 35.14	2/20/59	10.7 ± 4.3	6
Controls	"	3.3 ± 1	8

* Stand. dev.

† In these groups, rats inj. with normal plasma are used as controls; other control groups are uninj. rats.

TABLE II. Effect of 15 Minutes Boiling on Erythropoietic Activity of the Plasma.

Material inj.	Quantity inj. daily, cc	% Fe^{59} uptake	No. of recipients
Normal plasma	2	6.5 ± 3.6*	10
—after boiling	2	6.3 ± 6.2	"
"Anemic" plasma	2	15.1 ± 3	"
—after boiling	2	10.6 ± 4.9	9
"Anemic" plasma	2	30 ± 4.7	"
—after boiling	2	18.6 ± 6.8	10

* Stand. dev.

Fig. 3 shows that no activity was measured in plasma when hematocrit was 16% or more, some activity appeared when hematocrit reached 12%, and a sharp increase in erythropoietic stimulating factor occurred with lower hematocrits. The dose-response curve published by Garcia and Van Dyke(15) indicated that uptake of 30% required 5 times as much erythropoietin as uptake of 15%. Thus the erythropoietic factor response in the dog after bleeding is more striking than appears at first sight.

Four of the 6 dogs died from anemia; it is interesting that the most active plasma obtained was the last sample taken before death. When dogs were made severely anemic in 6 hours by replacing blood with dextran several times, no erythropoietic activity could be observed in plasma at the 6th hour.§ Possibly anemia of some duration is necessary to obtain demonstrable erythropoietin titer.

Like rabbits(11) and human beings(7,8), the dog secretes erythropoietin in urine when severe anemia exists. A very low hematocrit (9% or less) must be reached before activity is found in unconcentrated urine (Fig. 2). Since anemia of this degree is almost incompatible with life, this may explain why erythropoietic factor is seldom found in urine of anemic patients.

Erythropoietic factor may appear in urine as a result of glomerular filtration or tubular secretion. The latter mechanism is not unlikely since the role of dog kidney in erythropoietin production has received experimental support(16,17).

Boiling for 15 minutes reduces erythropoietic stimulating activity of plasma; Fe^{59} incorporation was reduced from 30% to 15% which is an 80% loss of activity according to dose-response curve. Stohlman(18) demonstrated a similar loss of activity when erythropoietically active rabbit plasma was boiled.

Active plasma and urine are obtainable from bled dogs and provide a source of erythropoietically active material for both biological and chemical investigation. It must be emphasized that this material is unstable even in frozen state. Lyophilizing it may prevent loss of activity.

Summary. Active erythropoietic factor is demonstrated in plasma and urine of bled dogs. A very striking erythropoietic factor response is obtained when severe anemia is produced. Activity is partially destroyed by boiling and by prolonged storage in freezer.

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§ Unpublished data.

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