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Received January 8, 1963. P.S.E.B.M., 1963, v112.

Immunological Neutralization of Various Erythropoietins.* (28148)

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Serum from rabbits immunized with an extract of erythropoietically active human urine will neutralize *in vitro* the erythropoietic activity of such extracts. Also, injections of such immune serum into normal mice will depress erythropoiesis, indicating that endogenous erythropoietin of mice can also be neutralized and that normal erythropoiesis in mice is erythropoietin dependent. Both neutralizations are considered to be the result of an antigen-antibody reaction directed against either erythropoietin or the general class of molecules to which erythropoietin belongs (1,2).

Erythropoietically active material has been found in plasma and in urine of several animal species. Some of these erythropoietins have been effective in stimulating erythropoiesis when injected into animals which differ in species from the source of the administered erythropoietin(3). The purpose of the present investigation is to study, using immunological methods, the interspecies relationships of erythropoietin.

Materials and methods. An extract of the urine of a patient with aplastic anemia was prepared by ultrafiltration through a collodion membrane. This extract had erythropoietic activity equivalent to approximately 20 cobalt units per milligram dry weight. An alum precipitate of the urinary extract was prepared by the method of Kabat and Mayer (4). Rabbits were immunized with a suspension of the alum precipitated urinary erythropoietin containing 1.5 mg of extract per ml. The rabbits were immunized by intravenous injection 3 times a week, following an initial injection given intraperitoneally. The volume of the alum precipitated suspension injected varied as follows: 3 injections of 1 ml; 3 injections of 1.5 ml; 4 injections of 2 ml; 4 injections of 3 ml; and 2 injections of 5 ml. The animals were bled on the fifth to seventh day after final injection and the serum collected. The ability of such serum to neutralize erythropoietin was determined by its effect in depressing the 24-hour red blood cell incorporation of Fe^{59} in normal mice after 4 daily subcutaneous injections of 0.25 ml of serum per day. The immune serum used in this study depressed the Fe^{59} incorporation to 1.5% from a value of 26.9% for mice similarly treated with normal rabbit serum.

An hypoxic environment or production of a severe anemia by injection of phenylhydrazine was used to elevate the serum erythropoietin levels. Groups of rats and rabbits and a single sheep were exposed in a decompression chamber to an oxygen tension equivalent to that of 20,000 ft altitude (approximately $pO_2 = 72$ mm Hg). All animals were bled within 30 minutes after 24 hours of hypoxic exposure; the sheep was bled again after 48 hours of exposure. Anemia was produced with phenylhydrazine in rabbits and mice. Rabbits were injected daily for 6 days with a solution of 2.5% phenylhydrazine hydrochloride, 2 ml on the first day and 1 ml on each subsequent day, and then bled the day following the last injection. Mice were given 4 injections of 1.5 mg phenylhydrazine hydrochloride per injection over a 6-day period and bled on the seventh day. Mice with transfusion-induced polycythemia were used for erythropoietin assay using a procedure slightly

* Supported in part by U. S. Atomic Energy Commission and in part by Cancer Research Funds of the University of California.

modified from DeGowin *et al.*(5). Groups of 10 female Swiss mice weighing 20-25 g were given 1 ml of washed red blood cells intraperitoneally on 2 successive days. On the fifth day following the last transfusion the test material was given subcutaneously. Two days later approximately 0.5 μc of Fe^{59} (approximately 0.05 μg of iron) was given intraperitoneally. The mice were bled 3 days after injection of the radioiron and the radioactivity of 0.5 ml of blood was determined. Values from any animal losing weight or whose hematocrit was less than 55% at time of autopsy were discarded. Results are expressed as percent of the injected dose of Fe^{59} in the calculated total blood volume. Blood volume of these hypertransfused mice was assumed to be 7.0% of total body weight.

Neutralization of erythropoietin in the various animal sera was determined by comparing erythropoietin activity after mixing and incubating 9 ml of each serum with 2 ml of the serum obtained from the rabbits immunized with erythropoietin (hereafter referred to as anti-E) or with 2 ml of normal rabbit serum. Treated sera were incubated at 37°C for 1 hour, then placed in a refrigerator (10°C) overnight. After centrifugation, 1 ml of the supernatant was injected subcutaneously into each assay mouse. The ability of the anti-E to neutralize an extract of erythropoietin obtained from phenylhydrazine anemic sheep plasma† was also determined. This extract was dissolved in 9 ml of saline, and the neutralization was performed in the same manner as for the various sera. Similar neutralizations were carried out using serum and urinary extract from the patient whose erythropoietin was used for immunizing the rabbits. The various sera were kept frozen until ready for use.

Results. Increased amounts of erythropoietin were found in sera taken from rats, rabbits, and a sheep exposed to a simulated altitude of 20,000 ft. The amount of erythropoietin found in serum of the 3 species after the same duration of exposure to altitude was noticeably different. The largest amount of erythropoietin was found in serum of the rat and the smallest in serum of the sheep. The data presented in Table I indicate that erythropoietic activity of the sera of these animals was completely neutralized by anti-E.

TABLE I. Neutralizing Effect of Immune Serum on Erythropoietic Activity of Sera from Various Species.

Source of serum	% radioiron incorporation in calculated total red cell volume: Normal rabbit serum	Anti-E serum
Rat: Altitude (24 hr at 20,000 ft)	37.46 ± 1.85*	.28† ± .03
Sheep: Altitude (24 hr at 20,000 ft)	2.67 ± .63	.17‡ ± .02
Altitude (48 hr at 20,000 ft)	3.78 ± .99	.20‡ ± .05
Rabbit: Altitude (24 hr at 20,000 ft)	11.36 ± .89	.38† ± .07
Phenylhydrazine-treated	13.56 ± 1.41	1.41† ± .34
Mouse: Phenylhydrazine-treated	25.90 ± 2.66	.16† ± .01
Human: Aplastic anemia	15.05 ± 1.50	.52† ± .11
Saline control	.18 ± .04	

* Standard error of mean.
† P value <.001 } Significance between respective
‡ P value <.01 } sera treated with normal rabbit serum and anti-E.

Increased amounts of erythropoietin were found in the sera of mice and of rabbits after injection of phenylhydrazine. The data presented in Table I also indicate that the erythropoietic activity of sera of phenylhydrazine-treated mice was completely neutralized by incubation with anti-E. Similar treatment of sera obtained from phenylhydrazine-injected rabbits caused a significant neutralization of the erythropoietic activity of such sera. However, in this case, some erythropoietin remained unneutralized, since radioiron incorporation into red blood cells was significantly higher than for saline-injected controls, with a P value of 0.001.‡

Erythropoietic activity of serum obtained from the aplastic anemic patient, whose urine was the source of erythropoietin used for immunizing the rabbits, was completely neutralized by incubation with anti-E (Table I).

Neutralization by anti-E is shown in Table II for erythropoietic activity of extracts made from urine of an aplastic anemic patient, and

† A1-0336, No. K103194A obtained from Hematology Study Section, Nat. Inst. Health.

‡ Fisher's table of T.

TABLE II. Neutralizing Effect of Immune Serum on Erythropoietin Extracts.

Extracts	% radioiron incorporation in calculated total red cell volume	
	Normal rabbit serum	Anti-E serum
Phenylhydrazine anemic sheep plasma	13.51 ± 1.36*	3.91† ± .50
Human urinary erythropoietin	11.88 ± 1.11	.28† ± .07
Saline control	.18 ± .04	

Approximately 1 cobalt unit of each extract was given to each assay animal.
* Standard error of mean.
† P value .001 (Significance between extracts treated with normal rabbit serum and anti-E).

from plasma of sheep made anemic by injections of phenylhydrazine. Erythropoietic activity of the urinary extract was completely neutralized. This confirms our previous findings for erythropoietic extracts from the urine of a different patient(1). Erythropoietic activity of sheep plasma erythropoietin was significantly, but not completely, neutralized by anti-E. Unneutralized erythropoietin caused a radioiron incorporation which was significantly higher than observed in the saline-injected controls ($P<0.001$).

Discussion. The present investigation demonstrates that the biological activity of erythropoietins found in serum of mice, rats, rabbits, sheep, and humans can be neutralized by addition of serum obtained from rabbits immunized with human urinary erythropoietin. Significant neutralization of erythropoietic activity was observed in all cases. In all but 2 instances it was complete. In these 2 cases phenylhydrazine anemia was used as a stimulus for increased erythropoietin levels. This may be of importance; however, other factors may be involved such as small species differences in the erythropoietin molecule. The results suggest that erythropoietins from the various animal sources investigated here are similar.

Rabbit anti-E was able to neutralize rabbit serum erythropoietin *in vitro*. This raises an interesting question. Was there neutralization of endogenous erythropoietin in the rabbits being immunized? It was noted at time of harvesting the antiserum that most of the rabbits having high anti-E titers had hematocrits well below normal. This suggests that some neutralization of endogenous erythropoietin in the rabbits being immunized did, in fact, occur, *i.e.*, an *autoimmune* condition results. A more extensive analysis of erythropoiesis in the rabbits being immunized is in progress.

Summary. Increased serum erythropoietin levels were produced in mice, rats, rabbits, and a sheep in response to hypoxia or phenylhydrazine anemia. In all cases, including that of the rabbit, the serum erythropoietin was neutralized on addition of serum from rabbits previously immunized with an erythropoietin extract of human urinary origin. Also, an erythropoietin extract prepared from phenylhydrazine anemic sheep plasma was neutralized by such serum. These data suggest that erythropoietins of the various animal species studied are similar.

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Received October 12, 1962. P.S.E.B.M., 1963, v112.