Factors Stimulating Erythropoiesis in Frogs

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HYPOXIA AND ANEMIA have been known for many years to stimulate erythropoiesis in mammals, including man.[1] In recent years, these stimuli have been shown to have their effect through a humoral factor, erythropoietin.[2] Jacobson, et al. have suggested that lack of oxygen, relative to the metabolic needs of the animal, is the fundamental stimulus for erythropoiesis and that this relative lack is sensed by some portion of the body, resulting in the production of erythropoietin.[3] The lack of oxygen in the sensitive tissues may be due to hypoxic environment, to reduction of circulating hemoglobin, to the histotoxic effects of chemicals or, on the other hand, to an increase in tissue oxygen needs due to elevation of the rate of metabolism.

There is some evidence that this hypothesis may not hold for lower animals. Chronic hypoxia does not induce polycythemia in turtles,[4] frogs[5,6] or lizards,[6] although this response is said to occur in newts[7] and fish.[8] No reticulocytosis is seen in response to acute or chronic anoxia in the turtle although evidence of increased erythropoiesis is seen in response to bleeding.[4]

The present experiments were designed to test the erythropoietic response of frogs to bleeding, hypoxia, and to cobaltous ion administration. The response was measured by determining the incorporation of thymidine-2-C^{14} into circulating erythrocytes. This compound is incorporated into desoxyribonucleic acid only of dividing cells; is not exchanged, once incorporated, until the cell dies; and is not incorporated into the non-dividing cell.[9] This same system was used to test the erythropoietic effect of various sera.

MATERIALS AND METHODS

Male frogs, Rana pipiens,* weighing 20–50 Gm. were kept in cages, permitting them access to water and dry area, for 2 weeks after receipt before being used. One hundred mg. of tetracycline were added to the water for the first 3 days in order to prevent "red leg." The frogs were fed earthworms and meal worms three times a week during the acclimatization period.

When the effect of hypoxia and bleeding on erythropoiesis was being tested, the frogs were divided into three groups on the first day of the experimental period. Those used to test the effects of bleeding were bled approximately 0.6 ml. (about one-third of their blood volume) by percutaneous cardiac puncture. Those used to test the effects of hypoxia were placed in a closed cage within which the degree of oxygen saturation could be reduced and controlled. Compressed air was added to pure nitrogen in such proportion as to maintain the desired degree of hypoxia, and the oxygen content of the air within the cage was monitored with a magnetic oxygen analyzer.† The frogs were maintained in the hypoxic atmosphere for periods of 16 hours on 4 successive days. In the first experiments,

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the oxygen content was maintained at 3 volumes per cent. A further reduction of the oxygen content to 1–1.5 volume per cent was tested in a second series in an effort to determine the effect of a maximal tolerable hypoxic stress. It was found that if the oxygen content were reduced below 1 volume per cent, 50–75 per cent of the frogs died during each exposure period of 16 hours. At a level of 1–1.5 volume per cent, the frogs were motionless, had contracted pupils, and relaxed the hind legs in a peculiar manner. These findings were also noted just prior to death in those frogs which died.

The third group of frogs served as negative controls.

When the erythropoietive effects of different sera and of cobaltous ion were being tested, the frogs were given intraperitoneally 0.25 ml. of the test material daily for 4 days. The serum of anemic frogs was obtained from 250–300 Gm. bull frogs, Rana catesbiana,[*] which were bled by cardiac puncture approximately 8 ml. on one day, 4–6 ml. on the day following and were terminally bled by decapitation on 2 days after the second bleeding. Their hematocrits at the time of the final bleeding were from 4–15 per cent (normal for the frog = 28 per cent).

Human serum known to contain large amounts of erythropoietin was obtained from a patient with aplastic anemia. Control sera consisted of serum from non-anemic patients or frogs. Fifty mg. of cobaltous chloride ($CoCl_2 \cdot 6 \, H_2O$) were dissolved in 10 ml. of 0.147 M NaCl; a total of 1 ml. of this solution was given in four equal daily doses.

Five days after the bleeding, first exposure to hypoxia, or first injection of test material, and at least 7 hours after termination of the last exposure to hypoxia, the frogs were given intraperitoneally 3 μc. of thymidine-2-C^{14}[†] in 0.5 ml. of 0.147 M NaCl. Ten days later the frogs were bled by decapitation into 1–2 drops of heparin (10,000 units per ml.). Microhematocrits were estimated on freely flowing blood. The heparinized blood was mixed with one part 0.147 M NaCl and two parts 6 per cent dextran (molecular weight approximately 188,000) in 0.147 M NaCl and the mixture was spun slowly for 5 minutes. The cells were distributed into three layers. The upper two, containing most of the leukocytes and thrombocytes and some erythrocytes, were removed and discarded. The lowest layer, containing mainly erythrocytes, was placed in a Wintrobe hematocrit tube and centrifuged at 6000 rpm for 1 hour. The supernatant and buffy layer were removed and discarded, and the remaining cells were diluted with 2 volumes of 0.147 M NaCl. After thorough mixing, the hematocrit of the mixture was estimated with a microhematocrit centrifuge. A drop of the mixture was spread on a glass slide according to standard technics for differential counting; this was stained with Wright's stain and the absence of leukocytes and thrombocytes confirmed. Twenty cu. mm. of the diluted cells were plated on aluminum planchettes 1¼ or 2 inches in diameter and dried with an infrared lamp. Preliminary experiments had shown that cells prepared in this manner had negligible internal absorption of beta radiation and the preparation could be considered infinitely thin for counting purposes. The radioactivity due to C^{14} was estimated in a low-background, gas flow beta emission spectrometer. Maximum counting error was ± 15 per cent (95 per cent confidence limits). The results are expressed as counts per minute per ml. of erythrocytes per μc. injected, corrected to a standard body weight of 40 Gm.

The assay of erythropoietin in the polycythemic mouse was performed according to a previously published method.[10]

Carbon dioxide excretion by the frog in normal and hypoxic atmosphere was measured by precipitating it as $BaCO_3$. Air or hypoxic gas mixture was passed through a tube containing sodium hydroxide on asbestos (Ascarite) in order to absorb out carbon dioxide. The gas was then passed into a 200 ml. closed bottle containing the frog in a small amount of water, whence it was conducted by plastic tubing into a carbon dioxide collecting tower. In this apparatus, the gas was bubbled through a sintered glass tube into 250 ml. of CO_2-free 1 N NaOH which trapped the CO_2 as Na_2CO_3. The flow of gas was maintained for

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[*]Obtained from The Lemberger Co., Oshkosh, Wis.
[†]Obtained from New England Nuclear, Inc., Boston, Mass.

Table 1.—*The Effect of Bleeding, Severe Hypoxia and Cobaltous Ion Administration on Erythropoiesis in the Frog*

Group	Number of Animals	Average Hematocrit	Incorporation of C^{14} (cpm/ml. RBC/μc. injected)	
			Mean	S.E. of mean
Bled	8	21.4	3008.9	716.0
Hypoxia				
3 volume per cent	11	29.9	38.2	11.9
1 volume per cent	7	27.7	41.5	4.03
Cobalt	6	34.5	55.8	10.3
Control	15	29.9	46.9	4.9

16 hours at a rate of 50 ml. per minute as monitored by a gas flowmeter. The oxygen content of the gas mixture was intermittently monitored with a magnetic oxygen analyzer. Blank experiments to determine the amount of carbon dioxide, due to factors other than the frog, were done each time by substituting 50 ml. of water for the frog.

The amount of carbon dioxide trapped in the NaOH was estimated by precipitating it as $BaCO_3$. Twenty-five ml. of 35 per cent NH_4NO_3 and 25 ml. of 30 per cent $BaCl_2$ were added to the 1 N NaOH. The resulting precipitate was collected on a tared sintered glass filter funnel by suction and was washed with 400 ml. of distilled water to dissolve any $Ba(OH_2)$ present. The precipitate was dried to constant weight (4 hours or more at 120 C.), re-weighed and the amount of $BaCO_3$ calculated. The carbon dioxide excretion was calculated as cu. mm. of CO_2 excreted per hour per Gm. body weight according to the following formula:

$$CO_2/hr./Gm. = \frac{(net\ wt.\ BaCO_3) \times (volume\ 1\ mole\ CO_2)}{(molecular\ wt.\ BaCO_3 \times (hours\ exposed) \times (body\ wt.)}$$

RESULTS

The incorporation of thymidine-2-C^{14} into the erythrocytes of control, hypoxic, bled, and cobalt-treated frogs are compared on table 1. The reduction of the mean hematocrit from 31.4 per cent to 21.9 per cent in the group of bled frogs resulted in a 70-fold increase in the incorporation of thymidine-2-C^{14} into the circulating erythrocytes. On the other hand, two levels of hypoxia, the lower, nearly fatal, resulted in no increase in erythropoiesis as measured by this criterion. Likewise, the administration of cobaltous ion, a stimulator of erythropoiesis in mammals, resulted in no significant increase in erythropoiesis.

As seen in table 2, the injection into normal frogs of serum from bled frogs results in a significantly greater incorporation of thymidine-2-C^{14} into red blood cells than the injection of serum from normal frogs ($t = 1.88$, $p = 0.05$ by Student's t test [Fisher]). However, the injection of the serum of an anemic patient did not significantly increase the incorporation of labeled thymidine into the frog's erythrocytes; one tenth of the dose given to these frogs increases the incorporation of Fe^{59} into the red blood cells of the polycythemic mouse by about 80 times that of normal human serum (negative control). When tested in the same mouse assay system, serum from bled frogs does not increase the incorporation of Fe^{59} into mouse erythrocytes.

Table 2.—*The Effect of Various Sera on Erythropoiesis in the Frog and Mouse*

Frog Assay

Material assayed	Number of animals	Mean hematocrit	Dose	Incorporation of C¹⁴ (cmp/ml. RBC/μc. injected)	
				mean	S.E. of mean
Bled frog serum	15	24.0	1.0 ml.	119.9	23.3
Normal frog serum	7	23.9	1.0 ml.	58.7	7.9
Anemic human serum	5	25.0	1.0 ml.	43.1	7.9
Normal human serum	—	—	—	—	—

Polycythemic Mouse Assay

Material assayed	Number of animals	Mean hematocrit	Dose	Per cent incorporation of Fe⁵⁹ in 1 ml. whole blood	
				mean	S.E. of mean
Bled frog serum	8	66	1.0 ml.	0.11	0.02
Normal frog serum	7	71	1.0 ml.	0.09	0.02
Anemic human serum	9	67	0.1 ml.	7.98	1.00
Normal human serum	8	65	1.0 ml.	0.12	0.03

DISCUSSION

The marked difference between the effect of anemia and of hypoxia on erythropoiesis in the frog contrasts sharply to the potency of both as stimuli for erythropoiesis in mammals.[1] This difference has also been noted for the turtle by Altland.[4]

It could be argued that in states of environmental hypoxia, the metabolic rate of the frog is proportionally reduced. This occurs throughout the range of oxygen tension in many subvertebrate forms, but decreases in metabolic rate are seen in only markedly reduced partial pressure of oxygen (below 30 mm.) in most vertebrates, including the frog.[11] For the frog, a six-fold reduction in oxygen tension did not alter the carbon dioxide output (see table 3). Altland has observed a similar phenomenon for the turtle.[4] Hence, it appears that for the conditions of the present experiment, the oxidative metabolism is not grossly altered.

It might be questioned whether the degree of hypoxia used was sufficient to bring about the stimulation of erythropoiesis. The tolerance of poikilothermic animals to extreme hypoxia is well documented.[6] Also, the oxygen dissociation curve for frog hemoglobin is far to the left of most mammals—that is, frog hemoglobin has a greater affinity for oxygen and hence requires lower levels of environmental oxygen to bring about desaturation than is seen with most mammalian hemoglobins.[12] However, the levels of hypoxia used were such that according to published data on oxygen dissociation characteristics of frog hemoglobin, the hemoglobin was 25–50 per cent desaturated. Further, since the level of hypoxia used in the second series (1–1½ volume per cent) was just above the fatal level for the frog, if environmental hypoxia is a stimulus to erythropoiesis in the frog, it is not so in the physiologically tolerable range. But even more important, this does not explain the great difference between the erythropoiesis-stimulating effect of bleeding and the lack of such an effect

Table 3.—*Carbon Dioxide Excretion by Normal and Hypoxic Frogs*

	Oxygen Content of Air	Number of Animals	Mean CO_2 Excretion (cu.mm./hr./Gm. body wt.)	S.E. of Mean
Control	20 vol. %	10	88.29	7.03
Hypoxic	3 vol. %	10	77.29	5.08

in response to marked hypoxia. It appears likely, therefore, either that the erythropoiesis-stimulating effect of bleeding is brought about by means other than consequent tissue hypoxia, or that the hypoxia induced by anemia is somehow different from that induced by changes in the environmental oxygen tension, or that somehow the poikilothermic animal makes adjustments to environmental hypoxia that are not made to anemia. These suggestions are contary to what is believed for mammals.

Cobalt is presumed to have its erythropoietin-producing effect through histo-toxic anoxia in sensitive tissues.[13] Since the frog does not have an erythropoietic response to cobalt administration in sub-toxic amounts,[14] this stimulus may be more like environmental hypoxia than anemic hypoxia in its fundamental mechanisms. Altland has found that cobalt is not an erythropoietic stimulus for the turtle.[15]

A humoral factor stimulating erythropoiesis, erythropoietin, has been demonstrated in the serum of many mammals, including man; but such factors have not been previously demonstrated in submammalian species. Human serum containing erythropoietin stimulates erythropoiesis in a wide variety of mammals, including the mouse.[2] Gordon has found that human urinary erythropoietin stimulates the uptake of Fe^{59} into the spleen of the urodele, *Desmognathus phoca*.[16] In the present studies, frog serum which stimulates erythropoiesis in other frogs does not do so in mice. Moreover, human erythropoietin which is active in the mouse does not stimulate erythropoiesis in frogs. These facts would indicate that the humoral factors stimulating erythropoiesis in frogs and mammals respectively were chemically different. Further characterization of the material from frogs must await a better system for assaying the activity in a quantitative rather than a qualitative way. These findings indicate that the response to bleeding by the production of a factor which stimulates erythropoiesis is phylogenetically quite primitive.

SUMMARY

1. Sublethal levels of hypoxia do not stimulate erythropoiesis in the frog as measured by the incorporation of thymidine-2-C^{14} into peripheral erythrocytes, whereas bleeding of approximately one-third of the blood volume increases erythropoiesis 70-fold as judged by this criterion. This is taken as evidence that for the frog, the fundamental stimulus for erythropoiesis may not be hypoxia.

2. Cobaltous ion administration does not increase erythropoiesis in the frog.

3. The serum of bled frogs increases the incorporation of thymidine-2-C^{14} into the erythrocytes of frogs into which it is injected. This serum does not

stimulate erythropoiesis in the polycythemic mouse. Serum from an anemic patient which contains large amcunts of erythropoietin as measured in the polycythemic mouse does not stimulate erythropoiesis in the frog.

SUMMARIO IN INTERLINGUA

1. Subletal grados de hypoxia non stimula le erythropoiese in ranas, secundo mesurationes per medio del incorporation de thymidina-2-C^{14} ad in erythrocytos peripheric. Del altere latere, secundo le mesme criterio, exsanguination de approximativemente un tertio del volumine de sanguine augmenta le erythropoiese septantuplemente. Isto es interpretate como evidentia que pro le rana stimulo fundamental pro le erythropoiese non pote esser trovate in hypoxia.

2. Le administration de iones cobaltose non augmenta le erythropoiese in le rana.

3. Le sero de sanguinate ranas augmenta le incorporation de thymidina-2-C^{14} in le erythrocytos de altere ranas ad in le quales illo es injicite. Iste sero non stimula le erythropoiese in muses poliycythemic. Sero ab un patiente con anemia, que contine grande quantitates de erythropoietina (secundo mesurationes in muses polycythemic), non stimula le erythropoiese in ranas.

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