The Renal Erythropoietic Factor (REF)
X. The Question of Species and Class Specificity* (34046)

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Erythropoiesis in mammals is under the control of a circulating erythropoiesis-stimulating factor (ESF or erythropoietin) (1) and the kidney is importantly concerned in its biogenesis (2, 3). A lack of species specificity for the ESF among members of the mammalian class is shown from observations that the ESF of human urinary or plasma origin can stimulate erythropoiesis in mice, rats, rabbits (1), and man (4). The question as to whether a mammalian type of ESF exists in lower vertebrates is not completely resolved. Reports have attested to the presence of an erythropoietic factor in the plasma of bled frogs (5), birds (6), and fish (7). However, attempts to stimulate erythropoiesis in mammals with anemic frog or bird plasma have proved unsuccessful (5, 6).

It has been demonstrated that a hypotonic extract of the light-mitochondrial fraction of rat kidneys contains a principle (renal erythropoietic factor, REF) which, when incubated with normal serum engenders the production of the ESF *in vitro* (3, 8). We have utilized this system to reinvestigate the question of species and class specificity among some representative animals of four classes of vertebrates.

Materials and Methods. All sera used were dialyzed against 0.005 *M* disodium ethylenediamine tetraacetate (EDTA) which eliminated the ESF-inactivating system reported earlier (9). Light-mitochondrial extracts were prepared from the kidneys of 50 hypoxic adult rats (Long-Evans strain), 2 hypoxic Dutch strain rabbits, 2 renal artery-ligated mongrel dogs, 2 normal pigs, 3 anemic sheep, 2 humans, 5 carps, 30 frogs (*Rana pipiens*), and 5 ducks. Hypoxia in rats and rabbits was induced by exposing the animals to 0.42 and 0.5 atm of air respectively for 16 hr. Anemia in sheep was achieved with phenylhydrazine injections followed by X-irradiation.[2]

Pig kidneys were secured from a slaughter house and processed within 2 hr of death. Human kidneys were obtained from two adult subjects, one a 24-year-old woman who died of barbiturate poisoning and the other a 65-year-old man whose death was ascribed to cerebral thrombosis. These light-mitochondrial extracts from all renal tissues were prepared as described earlier (10). Sera used were obtained from normal animals.

The incubation system consisted usually of 6 ml of dialyzed serum and 6 ml of the light-mitochondrial extracts (equivalent to 3 g of original kidney tissue). All incubations were open to the air and were conducted in a water bath incubator shaken at 37° for 60 min. The mixtures were assayed for erythropoietic activity in hypoxia-induced polycythemic mice (11). Five to six mice were injected ip with 2 ml of each sample and the activity estimated as the percentage of RBC-radioiron incorporation. Each experiment was conducted on two occasions and assayed separately with the exception of the experiments with human kidneys which were carried out

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TABLE I. Production of ESF *in Vitro* during Incubation of 6 ml REF and 6 ml Normal Serum from Six Mammalian Species.

Source of serum	Source of REF[a]					
	Hypoxic rat	Hypoxic rabbit	Dog	Pig	Anemic sheep	Human
Rat	11.90 ± 1.10[b]	8.31 ± 0.68	7.31 ± 1.14	6.11 ± 0.72	11.09 ± 0.81	9.39 ± 0.58
Rabbit	13.22 ± 2.08	7.13 ± 0.36	5.21 ± 0.81	5.91 ± 0.99	11.89 ± 1.26	6.08 ± 1.00
Dog	12.14 ± 1.16	5.91 ± 1.22	7.48 ± 0.92	6.78 ± 0.79	10.91 ± 2.11	5.55 ± 0.88
Pig	13.06 ± 2.78	5.64 ± 0.66	7.24 ± 1.52	5.09 ± 0.82	9.76 ± 0.64	7.55 ± 2.48
Sheep	10.86 ± 1.20	5.19 ± 0.48	6.69 ± 0.88	6.58 ± 1.29	12.16 ± 2.08	6.54 ± 1.11
Human	13.16 ± 3.36	7.42 ± 1.08	7.88 ± 1.68	5.74 ± 0.78	10.92 ± 1.98	7.44 ± 1.87
Controls						
	Saline	0.98 ± 0.36				
	NRS–EDTA[c] alone	1.22 ± 0.44				
	REF alone	0.89 ± 0.29				
	0.05 IRP U ESF[d]	7.18 ± 1.02				
	0.20 IRP U ESF	15.54 ± 1.36				

[a] Renal erythropoietic factor.

[b] Mean % RBC-^{59}Fe incorporated ± standard error of the mean for a 2-ml dose of incubate injected per assay mouse.

[c] Normal rat serum which was dialyzed against EDTA.

[d] Activity of ESF expressed in terms of International Reference Preparation Units. The ESF used was of human urinary origin and supplied by the Committee on Erythropoietin of the National Heart Institute.

once and assayed three times.

Results. The results are presented in Tables I and II. Light-mitochondrial extracts from kidneys of rats, rabbits, dogs, pigs, sheep, and humans were all capable of generating ESF when incubated with serum from the same species. In addition, incubation of the renal extract from one species with the sera of any of the other five species resulted in the generation of approximately the same quantities of ESF as when serum from the same species was used (Table I). These experiments therefore indicate that species specificity does not exist for either the REF or the serum factor, at least among the representatives of the Class Mammalia tested.

The possible presence of REF-like activity in hypotonic extracts of light-mitochondrial fractions of kidneys from selected members of nonmammalian vertebrate classes was investigated. The question of the existence of the substrate for the REF in the sera of these species was also examined. Table II indicates that incubation of renal extracts from carp, frogs, and ducks with sera of these species did not result in the generation of erythropoiesis-stimulating activity when tested in polycythemic mice. As before, the incubation of similarly prepared renal extracts from rats with equal volumes of rat serum, however, was accompanied by the appearance of considerable quantities of the ESF (Table II). The lack of ESF-generating capacity of the renal extracts from the 3 nonmammalian species was also shown from the failure of these extracts to produce the ESF when incubated with rat serum (Table II). To determine whether sera from these species contained the substrate for the REF, incubations consisting of hypoxic rat REF and sera from carp, frogs, and ducks were performed. Table II shows that while incubation of rat REF with EDTA-dialyzed sera from the carp and frog did not produce detectable activity, incubation mixture containing rat REF and EDTA-dialyzed serum from normal ducks evoked considerable erythropoietic activity in the test polycythemic mice.

Discussion. The presence of REF in the

TABLE II. *In Vitro* ESF-Generating Capacity of Light-Mitochondrial Extracts Obtained from Kidneys of Rat, Carp, Frog, and Duck.

Composition of incubation mixture	Mean % RBC–^{59}Fe incorp. ± SEM[a] for 2 ml of incubate injected per assay mouse
6 ml NRS-EDTA[b] + 6 ml rat LME[c]	9.05 ± 1.14
6 ml NFS-EDTA[d] + 6 ml frog LME	2.98 ± 0.42
6 ml NFS-EDTA + 6 ml rat LME	1.38 ± 0.32
6 ml NRS-EDTA + 6 ml frog LME	1.49 ± 0.35
6 ml NCS-EDTA[e] + 6 ml carp LME	2.21 ± 0.53
6 ml NCS-EDTA + 6 ml rat LME	2.49 ± 0.33
6 ml NRS-EDTA + 6 ml carp LME	1.29 ± 0.31
6 ml NDS-EDTA[f] + 6 ml duck LME	3.07 ± 1.35
6 ml NDS-EDTA + 6 ml rat LME	9.80 ± 1.53
6 ml NRS-EDTA + 6 ml duck LME	4.03 ± 1.17
Controls	
Saline	1.14 ± 0.29
0.05 IRP U ESF	7.51 ± 1.38
0.20 IRP U ESF	15.15 ± 2.65

[a] Standard error of the mean.
[b] EDTA-dialyzed normal rat serum.
[c] Light-mitochondrial extract.
[d] EDTA-dialyzed normal frog serum.
[e] EDTA-dialyzed normal carp serum.
[f] EDTA-dialyzed normal duck serum.

hypotonic extracts of the light-mitochondrial fractions of kidneys from a number of mammalian species suggests that similar mechanisms of ESF production are operative throughout the Class Mammalia. The lack of species-specificity in the REF-serum interaction in this class of Vertebrates is in accord with the finding that species-specificity does not exist for the ESF (1). Although it appears that renal tissues from nonmammalian species lack REF activity, it should be noted that the renal extract-serum mixtures were assayed for erythropoietic activity in the polycythemic mouse. It is possible that an erythropoietic principle was generated in these mixtures capable of stimulating red blood cell production in the respective species but which was unable to augment erythropoiesis in mammals. In this connection, it has been shown that anemic frog plasma stimulated erythropoiesis in normal frogs but did not increase the rate of red blood cell production in the mouse (5). Conversely, doses of mammalian ESF which were active in the mouse failed to produce an effect in the frog. Similar findings were reported for the bird (6).

On the other hand, while small quantities of mammalian ESF were ineffective in the gourami fish, very large doses of this hormone stimulated red blood cell formation in this form (7). Therefore, the reported failure of mammalian ESF to stimulate erythropoiesis in birds and frogs (5, 6) may have been due to the use of insufficient amounts of this factor. Similarly, the finding that anemic frog and bird plasma did not evoke an increase in erythropoiesis in the polycythemic mouse may have been due to insufficient amounts of plasma administered. This may also explain the observed inability of renal extract–serum mixtures from these species to stimulate erythropoiesis in the assay mouse (Table II). It is conceivable that all vertebrate erythropoietins possess a common biologically active core but that slight differences in attached chemical groups are responsible for the observed class specificity. Thus giving very large doses of one type (7) may produce an effect in another class despite the existence of physiological class specificity. The fact that incubation of mammalian REF with serum from ducks resulted in the generation of erythropoietic activity detectable in the assay mouse indicates that mammalian type of substrate for the REF is present in the blood of birds. No substrate activity could be detected in the sera of the other two nonmammalian forms studied.

Additional research is required to validate the possibility that class-specific fish, amphibian, reptilian, and avian erythropoietins do actually exist. In this regard, experiments testing the erythropoietic effects of incubation mixtures of lower vertebrate kidney light-mitochondrial extracts and serum in their respective species are needed. In addition, the possibility that organs other than the kidney in lower vertebrates (*e.g.*, the liver) function as the source of erythropoietin-generating factors must also be examined.

Summary. Incubation of hypotonic extracts of the light-mitochondrial fraction of

kidneys from mammalian sources with sera of several vertebrate species resulted in the generation of erythropoiesis-stimulating activity only when duck or mammalian serum was used. Similar extracts of kidneys from carp, frogs, and ducks failed upon incubation with sera from the same species or from mammalian sources to generate an ESF active in the mammalian assay system. Incubation studies also revealed that no species specificity with regard to the REF-serum interaction existed among the members of the Class Mammalia tested.

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