Studies of the Renal Erythropoietic Factor Using a Hemagglutination-Inhibition System[1] (34253)

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The renal erythropoietic factor (REF), in the absence of added normal serum, has been shown to have only slight erythropoietic activity (1, 2). Such activity is, however, easily demonstrable after incubation of REF with the serum of normal animals. The REF acts enzymatically on a serum substrate to produce active erythropoietin (ESF). However, all assays of REF have been performed *in vivo* and the possibility exists that the serum of the assay mouse may provide the necessary substrate for REF to produce a small amount of ESF. It is also conceivable that REF contains small amounts of ESF or an inactive precursor of ESF. Further REF studies were, therefore, carried out by use of a hemagglutination–inhibition (HAI) system using a gamma-globulin extract of anti-ESF serum. The results indicate that REF is immunochemically different from ESF, and that, after incubation of REF with normal serum, ESF is generated.

Methods. The REF was extracted from the kidneys of five species of mammals (1). For rat REF, 110 ml of extract were obtained from 55 g of kidneys from Long-Evans rats (250–280 g) which had been rendered hypoxic by exposure to 0.42 atm of air for 19 hr. Part of the material was frozen and used immediately after thawing and the other part was lyophilized and kept in the freezer until used. The REF from other species was extracted from normal tissues using the same techniques. The incubation procedure involved adding REF, dissolved in saline, to equal amounts of normal rabbit serum (NRS) that had previously been dialyzed first against 0.005 *M* Na_2–EDTA and then against water at 4° (1). All test materials (REF–saline, NRS–saline, REF–NRS) were incubated for 30 min at 37° and assayed for ESF content by the *in vivo* and *in vitro* systems outlined below.

The anti-ESF serum used in these studies was produced by immunizing a white New Zealand rabbit with human urinary ESF which was conjugated to methylated rabbit serum albumin by the method of Sueoka and Cheng (3). The anti-ESF serum was absorbed with normal human serum and the titer was determined as previously outlined (4). The gamma-globulins were extracted by the method of Stanworth (5).

In vivo assays for ESF activity were accomplished by injecting the test materials into ex-hypoxic polycythemic mice (6). Five to eight mice were used to test each sample. Each mouse received either saline, ESF standard, serum, or REF-serum, as a single 2.0 ml ip injection on day 3 post-hypoxia. On day 5, 0.5 μCi ^{59}Fe in 0.2 ml of saline was injected iv and on day 7 the mice were killed and the % RBC radioiron incorporation measured.

For *in vitro* studies, a modification of the HAI system outlined by Lange *et al.* (4) was used. A 0.075 *M* phosphate buffer (pH 7.4) was used as the diluent of the sensitized sheep red blood cells in place of the 1% NRS diluted with 0.9% NaCl. Gamma-globulins were employed in place of whole antiserum. These modifications made it possible to use

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TABLE I. ESF-Generating Activity of REF Preparations as Measured in Polycythemic Mice.

	Mean RBC ^{59}Fe incorporation (% ± SEM)[a]
Saline	0.81 ± 0.11
ESF, 0.2 units	13.24 ± 2.40
REF[b] (lyoph.) + saline	2.91 ± 0.45
REF (fresh) + saline	2.09 ± 0.38
NRS[c] + saline	1.86 ± 0.60
REF (lyoph.) + NRS	11.90 ± 1.72
REF (fresh) + NRS	10.11 ± 1.20

[a] Standard error of the mean.

[b] Renal erythropoietic factor obtained from hypoxic rat kidneys (1.0 ml/mouse).

[c] Normal rabbit serum dialyzed against EDTA (1.0 ml/mouse).

this system without the addition of whole serum other than that included in test substances.

After incubation at 37° for 30 min, the test substances were placed in the hemagglutinating titering plates[2] and 25 μl of anti-ESF gamma-globulin added. After 30 min of incubation at room temperature, 25 μl of tanned formalinized sheep red blood cells sensitized with a purified human urinary ESF were added (4). The plates containing the diluted material were sealed with plastic tape, mixed, and kept overnight at room temperature. The next day the results were recorded and the immunochemical units of ESF calculated according to the method of Lange *et al.* (4).

Results. Table I records the results of the *in vivo* assay of REF preparations. As shown, ip injections of the REF alone evoked only a small increase in ^{59}Fe incorporation. Serum incubated alone and injected into the polycythemic mouse resulted in slightly less ^{59}Fe incorporation than did REF alone. However, incubation of the REF and NRS together for 30 min resulted in the generation of a considerable amount of ESF.

The results of experiments in which the HAI techniques were used are summarized in Table II. REF incubated with saline showed no ESF activity in the HAI system. Changing the source of REF (rat, human, rabbit, dog, or sheep) or the methods of storage (lyophilization or fresh frozen) did not inhibit the hemagglutinating ability of anti-ESF gamma-globulins. NRS after dialysis against EDTA and undialyzed normal human serum contained only a small amount of ESF (7–27 immunochemical milliunits of ESF/ml) as measured by HAI methods. However, REF (fresh or lyophilized) incubated with normal rabbit or normal human serum before testing, gave increased ESF levels (75–110 immunochemical milliunits/ml).

Discussion. Kuratowska *et al.* (7) perfused hypoxic kidneys of rabbits with Tyrode's solution. The perfusate, initially inactive, became erythropoietically effective after incubation with an alpha-globulin fraction of normal serum. In later experiments, Contrera and Gordon (8) obtained significant erythropoiesis-stimulating activity in hypotonic extracts of kidneys from hypoxic rats. Subcellular fractionation yielded a light mitochondri-

TABLE II. ESF Activity of REF,[a] Serum, and REF-Serum Incubates, as Measured in a Hemagglutination–Inhibition System.[h]

	No. of determinations	Av (milliunits[g]/ml)
REF (rat)[b] + saline	29	0
(rat)[c] + saline	8	0
(human)[d] + saline	9	0
(rabbit)[d] + saline	8	0
(dog)[d] + saline	8	0
(sheep)[d] + saline	8	0
NRS[e] + saline	8	7
NHS[f] + saline	8	27
REF[b] + NRS	26	80
REF[b] + NHS	8	75
REF[c] + NRS	8	110

[a] Renal erythropoietic factor.

[b] REF was obtained from hypoxic rats and used without storage.

[c] REF was lyophilized and stored until used.

[d] These materials were heat inactivated in a water bath at 50° for 30 min.

[e] Normal rabbit serum.

[f] Normal human serum.

[g] Values expressed as immunochemical ESF units.

[h] All materials were mixed together: equal parts REF and saline, NRS and saline, or REF and serum and incubated 30 min at 37° before assaying.

[2] Obtained from Cooke Engineering Company, Alexandria, Va.

al extract which had little or no activity when injected ip into assay mice. However, upon incubation of this fraction (later termed the REF) with normal serum for 30 min, significant ESF activity appeared in the reaction mixture (8, 9). Prolonged incubation of the REF with serum resulted in significant loss of ESF activity (10), but dialysis of the serum against EDTA followed by dialysis against water (1) circumvented this inactivation process and made it possible to investigate the kinetics of the REF–serum interaction. It was found (2) that the amount of serum substrate converted to ESF in a given time was proportional to the REF concentration. This time dependence followed first-order kinetics which suggested that the reaction was enzyme catalyzed. Indeed, other data (11) supported the view that the REF-substrate was produced by the liver, and was converted to the ESF by the kidney enzyme (REF). The product of REF and serum incubation had the same antigenic specificity as ESF (12).

The present experiments confirm and extend a number of the findings discussed above. The bioassays confirmed prior studies which showed that neither REF nor normal serum, when injected separately, caused a significant increase in ^{59}FE RBC incorporation in polycythemic mice. However, incubation of REF and serum together resulted in a marked increase in ^{59}Fe RBC incorporation in the assay mice. In the *in vitro* HAI system, REF by itself did not inhibit the hemagglutination of sensitized sheep RBC by gamma-globulins extracted from anti-ESF serum. As previously shown (4), and confirmed in these studies, normal serum inhibited HA and thus demonstrated the presence of some ESF. The greater degree of inhibition of HA of the sensitized sheep cells by the incubated mixture of REF and serum indicated the further generation of ESF. As shown herein REF differs immunochemically from ESF. Therefore, the data support the contention that REF is probably not an inactive precursor of ESF. On this basis and from other evidence (2) REF is probably an enzyme that acts upon a serum substrate to produce the ESF.

The kidneys of rats, rabbits, dogs, sheep, pigs, and humans contain REF (13). In the present experiments, the testing of REF preparations without the addition of serum from different species in the HAI system indicated that no ESF was present. The REF from the kidneys of several different species has, however, been shown (13) to react with serum from the same or other animals.

The use of hemagglutination techniques has provided an additional tool for ESF studies. The HAI technique was employed to determine the quantities of the ESF in normal human serum (4). A range of 7–30 immunochemical milliunits of ESF/ml of serum was found, a result which agrees with the values found in the present experiments for normal human or normal rat serum (7–27 immunochemical milliunits of ESF/ml). The HAI techniques can be used for detecting low levels of the ESF in human sera, rabbit sera, ESF extracts, and perhaps other ESF-containing materials. If these findings are substantiated by other investigators, it seems reasonable that in the future, use will be made of this technique in experiments requiring a rapid and sensitive test for ESF.

From the present results, the inference may be drawn that the small increase in ^{59}Fe incorporation in the red blood cells of polycythemic mice treated with the REF as compared to the saline-injected controls probably is due to ESF generated endogenously in the test mouse. Also, it is clear that the REF acts on a serum component to produce a factor that is immunochemically and biologically similar to ESF.

Summary. A hemagglutination–inhibition system utilizing gamma-globulins extracted from anti-ESF serum for the determination of erythropoietin (ESF) is reported. Use of this technique showed that the renal erythropoietic factor (REF) is different immunochemically from ESF. Small but detectable levels of ESF were found in normal serum and the incubation of REF with serum resulted in the generation of ESF. The small increases in red cell ^{59}Fe incorporation noted in assay mice treated with the REF alone is probably due to the endogenous production of the ESF.

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