

## Immunologic Aspects of the Biogenesis of Erythropoietin

By JOHN C. SCHOOLEY, ESMAIL D. ZANJANI AND ALBERT S. GORDON

CONSIDERABLE EVIDENCE, recently reviewed by Gordon, Cooper and Zanjani,<sup>1</sup> supports the concept that erythropoietin (ESF), the hormone regulating erythropoiesis, is produced or activated primarily by the kidney. Normal serum, which is incapable of stimulating erythropoiesis in the plethoric mouse, acquires erythropoietic-stimulating activity following incubation with a hypotonic extract of the light mitochondrial fraction of the normal or hypoxic kidney.<sup>2,3</sup> The kidney extract which has been termed the "Renal Erythropoietic Factor" or "REF" cannot by itself significantly stimulate erythropoiesis in plethoric mice. Kinetic studies of the REF-serum interaction suggest that the REF is an enzyme acting on a serum substrate to produce ESF.<sup>1,5</sup> We have previously demonstrated, using rabbit antibody against ESF, that the erythropoietic stimulation observed in plethoric mice after injection of incubated REF-serum mixtures is the result of ESF production *in vitro*, and not the result of some indirect action of the reactants resulting in the *in vivo* production of ESF.<sup>4</sup> Serum from rabbits immunized with ESF, which has been shown to neutralize the biological effects of ESF *in vivo* and *in vitro*,<sup>6-9</sup> was used in the present studies to demonstrate that the immunologic properties of REF and the serum substrate are different from those of the product of their interaction, ESF.

### MATERIALS AND METHODS

#### *Preparation of Antisera*

Anti-ESF was obtained from rabbits immunized with human urinary erythropoietin by a schedule previously described.<sup>8</sup> One ml. of this antiserum could neutralize approximately 25 I.R.P. units of ESF.

Goat antirabbit gamma globulin (GARGG) was obtained from goats immunized monthly with rabbit gamma globulin in complete Freund's adjuvant. Immunodiffusion in agar indicated only one precipitin line. Each milliliter of this antibody precipitated at least 4 mg. of rabbit gamma globulin. This immune serum was used as an anti-anti-ESF. In separate experiments it was determined that the addition of 2 ml. of this immune serum to the amount of anti-ESF used was sufficient to prevent its neutralizing action.

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Six rabbits were given multiple subcutaneous injections of 4 ml. of a 1:1 mixture of REF in saline and complete Freund's adjuvant followed by at least two similar injections using incomplete Freund's adjuvant. These immunizing injections were given at weekly intervals. Serum was collected two weeks after the last immunization. Precipitating antibodies against at least six separate proteins in the REF extract were observed by gel diffusion techniques.

#### *Preparation of REF and Serum*

REF was prepared from the kidneys of Long-Evans rats weighing  $\approx$  220–260 Gm. fifteen-and-one-half hours after exposure to a simulated altitude of 0.42 atm. as previously described.<sup>3</sup> Each milliliter of REF employed in the experiments using posthypoxic mice as assay animals was extracted from 2 Gm. of kidney, and in the other experiments from 1 Gm. of kidney.

Serum was obtained from normal adult rats and dialyzed against 0.005 M disodium ethylene diamine tetraacetate (EDTA) for 24 hours followed by another 24 hours of dialysis against deionized water in the cold.<sup>10</sup> This normal rat serum has been designated as NRS.

#### *Incubation Procedure*

Ten ml. of REF and 10 ml. of NRS were mixed and incubated in a water bath at 37°C for 45 minutes with shaking. All reaction vessels were open to the air. The various antibody additions were thoroughly mixed and were allowed to react for 15 minutes before centrifugation. Any immune precipitates formed as a result of antibody additions were removed by centrifugation. Total handling time was kept constant for all incubations. At the conclusion of the experiment, the reaction mixtures (less immune precipitates) were diluted to 26 ml. and stored in ice water until injected into the assay mice.

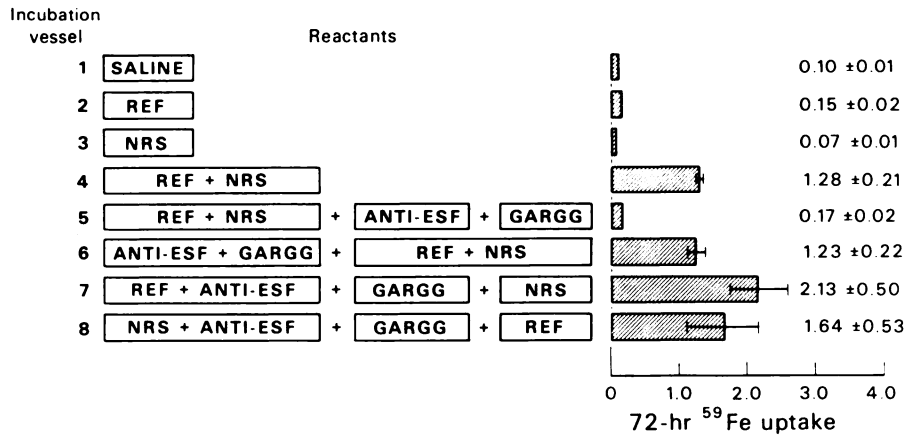
#### *ESF Assay*

The erythropoietic activity of the incubation mixtures was determined using either posthypoxic or transfusion-induced plethoric mice. Posthypoxic CF-1 mice were prepared by exposure to 0.42 atm. simulated altitude for 19 hours daily to a total of 219 hours. Three days after final removal of the mice from the chamber, the mixtures to be assayed were injected, and two days later <sup>59</sup>Fe was injected intravenously. The mice were bled 48 hours after the <sup>59</sup>Fe injection. Female C<sub>3</sub>H mice were hypertransfused with two consecutive intraperitoneal injections of 1 ml. of packed isologous red blood cells and were used for the assay five days later. Fifty-six hours after injection of the incubation mixtures, <sup>59</sup>Fe was injected intravenously, and 72 hours later the mice were bled. The <sup>59</sup>Fe in 0.5 ml. of blood was measured, and the results for both assays were expressed as per cent of the injected <sup>59</sup>Fe in the calculated blood volume which was assumed to be seven per cent of the body weight. In all experiments 2 ml. of the material to be assayed was injected intraperitoneally. Ten to 12 assay mice were used for each experimental group. The standard error of the mean is indicated.

### RESULTS

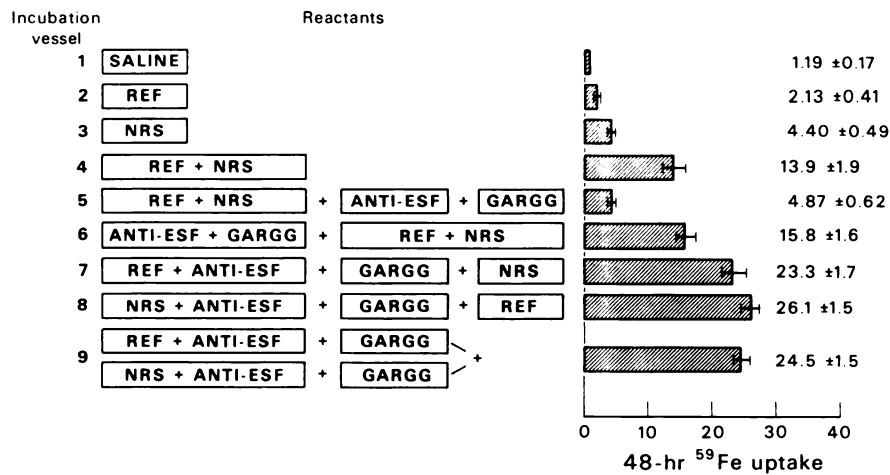
The results of the experiments are summarized in Figs. 1 and 2. In experiments reported in Fig. 1, erythropoietic activity was assayed using the transfusion-induced polycythemic mouse, and in Fig. 2 posthypoxic mice were employed. The general pattern of results was the same in both assay systems, but the magnitude of the <sup>59</sup>Fe uptake was about 10 times greater in the posthypoxic mouse. The various reactants are diagrammed in boxes, the sequence of their additions proceeding from left to right in both figures. The order of the various additions is critical. Little or no erythropoietic activity, compared to saline controls (vessel one), was observed when REF (vessel two) or NRS

## Transfused polycythemic mouse assay



(vessel three) was injected alone into assay mice. Following incubation of REF and NRS, however, a significant increase in erythropoietic activity could be measured in both assay systems (vessel four). This increased erythropoietic activity could be completely prevented if anti-ESF (enough to neutralize 12.1 I.R.P. units) was added to the mixture after incubation (vessel five). Any excess anti-ESF which had not combined with ESF, and probably anti-ESF which had combined with ESF was removed from the incubation mixture by the addition of goat antirabbit gamma globulin (GARGG) before injection of the mixture into the assay mouse. The appropriate amounts of anti-ESF and GARGG to be used were determined in separate experiments, but it is evident that the amounts selected were adequate since their addition before the incubation of REF and NRS did not affect the magnitude of the measured erythropoietic response (vessel six compared to vessel four). This indicates that anti-ESF was not introduced into the assay mouse after combination with

## Post-hypoxic mouse assay



**Table 1.—Effect of GARGG on Anti-ESF Removal**

	Posthypoxic Mouse 48 Hour <sup>59</sup> Fe Uptake
Saline	0.63 ± 0.14 *
0.05 I.R.P. u ESF	9.31 ± 1.10
(Anti-ESF + GARGG) + 0.05 I.R.P. u ESF	8.03 ± 0.62
0.20 I.R.P. u ESF	18.00 ± 1.40
(Anti-ESF + GARGG) + 0.20 I.R.P. u ESF	17.50 ± 1.40
(0.20 I.R.P. u ESF + Anti-ESF) + GARGG	1.89 ± 0.35

\* The standard error of the mean.

GARGG. The result of this incubation also demonstrates, as we reported previously,<sup>4</sup> that the ESF was generated in the test tube and not in the assay mouse.

Table 1 gives the data indicating that the amount of GARGG was adequate to remove anti-ESF completely, thereby preventing the neutralizing action of the latter on ESF.

It is evident that if anti-ESF is added to a tube and subsequently removed by a suitable amount of anti-anti-ESF (GARGG) and then a known amount of ESF added, the magnitude of the erythropoietic response of the assay animal is not significantly reduced. However, if the same amounts of anti-ESF and ESF are reacted, followed by addition of GARGG, the erythropoietic response of the assay animal is almost completely abolished.

The results of the incubations and the sequence of antibody additions shown in vessels seven and eight of Figs. 1 and 2, and vessel nine of Fig. 2 indicate that the generation of ESF by REF and serum is not significantly altered if anti-ESF is added to the individual reactants and subsequently removed by GARGG before incubation of the two reactants.

Incubation of REF extract with anti-REF before incubation with serum significantly increased the production of ESF compared to controls, as indicated in Table 2.

Injection of the same amount of anti-REF into assay mice did not increase the erythropoietic response compared to saline-injected controls. In addition, injection of 0.25 ml. daily of anti-REF into mice exposed to simulated altitude for five days did not decrease the magnitude of the erythropoietic response.

#### DISCUSSION

Zanjani, Gordon and co-workers<sup>1,10</sup> propose that an enzyme present in the REF preparations acts upon a presumably liver-produced substrate present

**Table 2.—Effect of anti-REF on ESF generating capacity of REF**

	Post-hypoxic Mouse 48 Hour <sup>59</sup> Fe Uptake
REF + NRS	13.90 ± 1.90 *
(REF + Anti-REF) + NRS	27.70 ± 1.40
Anti-REF	1.85 ± 0.56
Saline	1.19 ± 0.17

\* The standard error of the mean.

in the serum to produce ESF. The results of these experiments support the concept that active ESF is generated in vitro. The reaction may involve the splitting off of a portion of the protein substrate in a manner analogous to the renin-angiotension II system. The apparent failure of anti-ESF to combine with REF or the serum substrate tends to favor this concept and suggests that antigenic sites, probably on the serum substrate, become exposed to react with anti-ESF only after the action of the REF.

The failure of anti-REF immune serum to prevent the production of ESF either in vivo or in vitro is somewhat unexpected, but these preliminary results do suggest that such sera will have considerable usefulness in purifying and concentrating the active component of the crude REF fraction. Moreover, one experiment suggests that antibody against the active component in the REF extract may be present, although its effects are masked by the presence of other antibodies in the immune serum which remove inhibitors of the reaction resulting in an apparent increase in ESF production. An increased ESF production occurred after anti-REF addition. An  $^{59}\text{Fe}$  uptake at  $21.7 \pm 2.6$  per cent was observed when a more purified REF preparation was reacted with serum, whereas an  $^{59}\text{Fe}$  uptake of only  $9.57 \pm 2.76$  per cent was found when the same purified REF was combined with anti-REF that had been absorbed, previous to the incubation, with crude inactive REF. Further experiments may clarify these results.

#### SUMMARY

Immunologic evidence is presented that the interaction of Renal Erythropoietic Factor (REF) and a serum substrate results in the production in vitro of erythropoietin. Antibody capable of neutralizing erythropoietin (ESF) does not combine with either the REF or the serum substrate. Immune serum against the crude REF extract has been produced. This immune serum does not prevent the in vitro action or in vivo production of REF. This failure, however, may result from the diversity of antibodies produced.

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