Erythropoietic Effects of Bilirubin in Rats[1] (37373)

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Red blood cell hemolysates (1–3) and heme-related compounds (4, 5) have been reported to stimulate erythropoiesis in several animal species. Erslev (6) and Okamura, Udupa and Reissmann (7) reported recently that the erythropoietic effect of red cell hemolysates is due to the release of renal erythropoietin (ESF). Brown, Altschuler and Cooper (4) reported that bilirubin produced an increase in reticulocytes and iron incorporation in red blood cells of fasted dehydrated rats. However, the mechanism by which heme-related compounds stimulate erythropoiesis has not been studied.

The present studies were undertaken to clarify the mechanism of the erythropoietic action of bilirubin, an end product of red cell metabolism.

Materials and Methods. Female Sprague–Dawley rats weighing 250–275 g were used in these studies. Purified bilirubin (Sigma Chemical Co.) was dissolved in a solution containing 0.5% sodium carbonate and 0.52% sodium chloride and the pH was adjusted from 10.0 to 8.0 with 1.0 *N* HCl solution before injection. The bilirubin solution was injected subcutaneously into starved rats according to the method described by Fried *et al.* (8). The rats were weighed and the food was removed on the first day and throughout the experiment with water provided *ad libitum*. Bilirubin was injected on Days 2, 3 and 4 of the assay at doses of 1.0, 3.5 and 10 mg/100 g. On Day 5, each rat received 1.0 μCi of radioactive ferrous citrate (^{59}Fe) via the tail vein. Twenty-four hours later percent ^{59}Fe incorporation in red blood cells was determined.

To determine whether the effects of bilirubin on ^{59}Fe incorporation in red blood cells of starved rats was due to erythropoietin elaborated from the kidney, bilirubin was injected into normal and bilaterally nephrectomized rats. The rats were anesthetized with ether and nephrectomy was performed through a retroperitoneal incision. Each rat received a subcutaneous injection of 10 mg/100 g immediately after nephrectomy and 24 and 48 hr later. Six hours after the last injection of bilirubin the rats were bled via cardiac puncture and serum erythropoietin titers were determined in exhypoxic polycythemic mice (9).

The bilirubin concentration of the serum samples was measured by the method of Ducci and Watson (10) to determine whether the erythropoietic effects of rat serum on ^{59}Fe incorporation in red blood cells was due to residual bilirubin remaining in the serum.

To confirm that erythropoietic activity seen in the serum from the rats treated with bilirubin was due to erythropoietin, the neutralizing effect of erythropoietin antiserum (anti-ESF) on the serum samples was investigated. Anti-erythropoietin serum was produced in rabbits by injecting a mixture of human urinary erythropoietin and Freund's adjuvant into multiple sites (im, sc footpads and intradermally) 2 times/wk for 2 wk. Anti-ESF activity of the rabbit serum was determined *in vitro* via Ouchterlony gel diffusion for immunological reactivity (11) and in the exhypoxic polycythemic mouse assay (9) for biological neutralizing properties. The sera from normal and nephrectomized rats treated with bilirubin were incubated with erythropoietin antiserum at 37° for 45 min, injected into polycythemic mice sc and the percent

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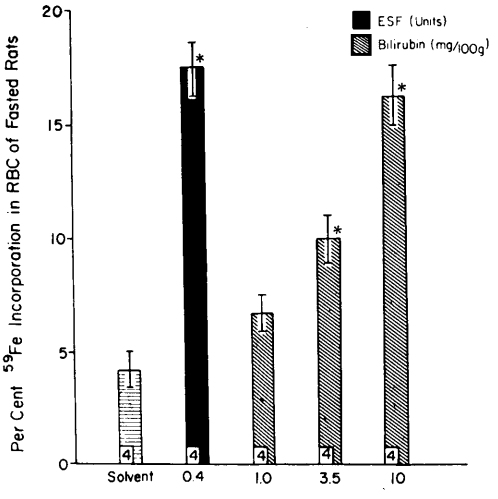

FIG. 1. Mean percentage ^{59}Fe incorporation in RBC of starved rats injected with solvent, human urinary erythropoietin or bilirubin. Number at the bottom of each bar represents the number of experiments (5 assay rats in each experiment). The standard error of the mean is shown at the top of each bar. (*) Significantly different ($p < 0.05$) from normal rats receiving solvent alone.

^{59}Fe incorporation in red cells was determined.

Results. Figure 1 illustrates the mean percentage ^{59}Fe incorporation in red cells of fasted rats (5 per group) treated with bilirubin. The values represent the mean of 4 different experiments. Doses of 1.0, 3.5 and 10 mg/100 g bilirubin gave a dose-related increase in percentage ^{59}Fe incorporation into red blood cells. The groups receiving 3.5 and 10 mg/100 g were significantly ($p < 0.05$) higher than that of the solvent controls.

Figure 2 demonstrates the percentage ^{59}Fe incorporation in red cells of exhypoxic polycythemic mice injected with serum from normal or bilaterally nephrectomized rats treated with bilirubin (10 mg/100 g). The serum from 5 rats was pooled and assayed in exhypoxic polycythemic mice (5 mice/assay). The ^{59}Fe incorporation values are the means of 6 or 7 different experiments. The mean percentage ^{59}Fe incorporation in red blood cell values of the polycythemic mice receiving serum from rats injected with solvent alone was 2.45 ± 0.84%. On the other hand, the activity of serum from normal rats treated with 10 mg/100 g bilirubin was significantly ($p < 0.05$) higher than that of the control rat serum. The ^{59}Fe incorporation values in polycythemic mice receiving serum from nephrectomized rats injected with solvent alone were not significantly different from that of polycythemic mice receiving serum from normal nonnephrectomized rats treated with solvent alone. However, serum from the nephrectomized rats injected with bilirubin showed a slight, but not significant, increase in ^{59}Fe incorporation in red blood cells of polycythemic mice.

Table I demonstrates the inhibitory effects of anti-erythropoietin serum (anti-ESF) on the erythropoietic activity of normal or nephrectomized rat serum in two different experiments. The ^{59}Fe incorporation values from two different experiments of polycythemic mice injected with serum from nephrectomized rats treated with bilirubin were less than that of intact rats. This erythropoietic activity was almost completely neutralized by incubation with anti-ESF.

Discussion. The present studies indicate that bilirubin produces an erythropoietic effect in rats and the primary mechanism by

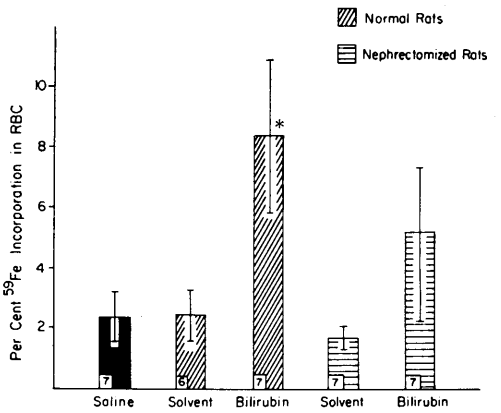

FIG. 2. Mean percentage ^{59}Fe incorporation in RBC of exhypoxic polycythemic mice (5 mice in each group) injected with serum from normal or nephrectomized rats treated with bilirubin (10 mg/100 g). Number at the bottom of each bar indicates number of experiments in each group and the standard error of the mean is indicated at the top of each bar. (*) Significantly different ($p < 0.05$) from the serum of normal rats injected with solvent alone.

TABLE I. Inhibitory Effects of Anti-ESF on Serum from Rats Treated with Bilirubin.[a]

	Expt. no.	^{59}Fe incorporation in RBC of polycythemic mice (%)	
		Serum	Serum + anti-ESF
Intact normal rats	1	4.49 ± 0.88[b]	0.82 ± 0.55
	2	8.51 ± 2.23	1.71 ± 1.22
Nephrectomized rats	1	1.65 ± 0.30	0.80 ± 0.09
	2	3.96 ± 1.55	1.34 ± 0.83

[a] Each rat received a dosage of 10 mg/100 g sc for 3 times.
[b] ± = standard error of mean.

which this compound stimulates erythropoiesis appears to be through an increase in endogenous erythropoietin production. Brown, Altschuler and Cooper (4) compared the erythropoietic effects of several heme-related compounds and found that the compounds with pyrrole rings produced a significant increase in ^{59}Fe incorporation in red blood cells of fasted dehydrated rats. The compounds with open ring structures such as bilirubin and biliverdin were found to be erythropoietically active.

Injections of bilirubin into normal rats were found in the present experiments to produce a significant increase in serum erythropoietin titers. This effect was partially blocked by bilateral nephrectomy. Total bilirubin in the serum samples from normal or nephrectomized rats, either treated with bilirubin or solvent alone, were not significantly different. Therefore, it does not seem likely that the erythropoietic activity of these sera is due to residual bilirubin remaining in the serum. In addition, the serum erythropoietic activity was almost completely inhibited by antiserum to erythropoietin.

Erslev (6) and Okamura, Udupa and Reissmann (7) reported that the erythropoietic effects of hemolysates of rat red cells was due to stimulation of the production of renal erythropoietin. These workers (6, 7) also reported that this effect was abolished by anti-ESF. It is interesting to note that in our experiments serum from nephrectomized rats treated with bilirubin showed slight erythropoietic activity. It is possible that the large dose of bilirubin used in our experiments may cause the elaboration of erythropoietin from extrarenal sources. However, it is still quite possible that this effect is due to a metabolite of bilirubin in the serum which could not be detected by our bilirubin assay method.

Brown, Altschuler and Cooper (4) postulated that the erythropoietic activities of hemolysates and heme-related compounds are related to a feedback mechanism in hemolyic anemia. Erslev (6) has also suggested that the effect of hemolysates on erythropoietin release from the kidney may be due to a histotoxic hypoxic mechanism or a direct effect on some renal site of ESF production.

Summary. Bilirubin was found to increase ^{59}Fe incorporation in red blood cells of fasted rats. A significant increase in ^{59}Fe incorporation in red blood cells of exhypoxic polycythemic mice was also seen following the injection of serum from normal rats treated with bilirubin. This effect was completely blocked by antiserum to erythropoietin, indicating that the effects of bilirubin is erythropoietin dependent. Nephrectomy was also shown to markedly antagonize but not completely block the erythropoietic effects of bilirubin in rats.

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