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Renal Mechanisms Underlying Actions of Androgen and Hypoxia on Erythropoiesis.* (31518)

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Previous reports have indicated that androgens exert a stimulatory effect on erythropoiesis in endocrine-deficient and intact animals(1,2,3). A resurgence of interest in this field has stemmed from more recent observations that testosterone may induce remissions in some patients with aplastic anemia(4) and with myelofibrosis(5).

Several modes of action of androgen on erythropoiesis have been proposed: 1. a direct effect upon the blood-forming organs (6,7); 2. potentiation of erythropoietin (ESF) action(8); 3. stimulation of production of the ESF(9,10).

Further evidence is presented here to support the concept that androgen stimulates production of ESF in rats and that this action is mediated through the renal erythropoietic factor (REF), a recently demonstrated principle which is not ESF but which when added to normal serum engenders the production of ESF(11,12,13,14). In view of reports (8,15) that animals rendered severely plethoric by hypertransfusion are less responsive to the erythropoiesis-stimulating effects of androgen, both normal and hypertransfused rats were employed in this study.

Materials and methods. Experiments with androgen. In all experiments, adult male rats (250-300 g) of the Long-Evans strain were employed as serum and kidney donors. One group of 5 rats received a single intramuscular injection of 12.5 mg long-acting testosterone cyclopentylpropionate (TCP) (Depo-testosterone, Upjohn Co.) in 0.25 ml cottonseed oil. The other group of 5, constituting the controls, was injected with 0.25 ml of the vehicle, cottonseed oil. Another group of 10 rats was rendered plethoric by intraperitoneal injections, on 2 successive days, of 10 ml of whole heparinized blood obtained from normal male rats. As with the non-plethoric groups, 5 of the plethoric rats were given a single intramuscular injection of 12.5 mg TCP while the other 5 received only the cottonseed oil. All injections were administered 4 days after the 2nd transfusion. Hematocrits at this time ranged from 65% to 70%.

Six days after the injections, all rats comprising the 4 groups were exsanguinated, the bloods allowed to clot at 5°C and the sera of the different groups pooled. The kidneys of the 4 groups were also separately pooled and subjected to the following procedure for extraction of the REF. The organs were weighed and minced. Ten ml of cold 0.25 M sucrose were added for each g of renal tissue. The mixture was homogenized in a Potter-Elvehjem homogenizer. Centrifugation was carried out at 6,300 $\times$ *g* for 15 minutes at 5°C and the sediment discarded. The sucrose supernatant was now recentrifuged at 21,000 $\times$ *g* for 20 minutes at 5°C. The sediment obtained ("light mitochondrial" fraction) was resuspended in 0.02 M phosphate buffer, pH 6.8 and the mixture immediately frozen. After thawing, the material was centrifuged at 37,000 $\times$ *g* for 30 minutes. The super-

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natant fluid which contains the REF was kept frozen until used(13).

The inclubation procedure consisted of adding one volume of the REF-containing supernatant fluid to 10 volumes of normal rat serum. All incubations were conducted in a water bath shaken at 37°C. Reaction vessels were left open to the atmosphere and the reactions were stopped at the end of a standard 15-minute incubation period by plunging the reaction flasks into ice water.

Experiments with hypoxia. One group of 5 normal adult male rats was subjected to 0.4 atmosphere for 16 hours. Another 5 rats served as untreated unexposed controls. As with the androgen-treated groups, an additional 10 rats were rendered plethoric by 2 successive daily intraperitoneal injections of homologous blood. Five of these were exposed to 0.4 atmosphere for 16 hours and the other 5 comprised the unexposed plethoric controls.

Immediately after the period of hypoxia, the exposed rats and the unexposed controls were exsanguinated and the sera and kidneys of each of the 4 groups collected and separately pooled. The kidneys were subjected to the procedure for the extraction of the REF and the incubations of the REF with normal rat serum conducted as described above.

Assay methods. All sera and REF-serum incubates were assayed for ESF activity in the hypoxia-induced polycythemic mouse (16). Five to 6 mice were used to test each sample. Each mouse received either 1.0 ml of serum or 1.0 ml of the incubate as a single intraperitoneal injection on day 3 post-hypoxia. On day 5, they were injected intravenously with a quantity of ^{59}Fe in 0.2 ml saline registering 150,000 cpm. They were killed on day 6 and the % RBC-radioiron incorporation estimated(16).

It has been pointed out(13,14) that intraperitoneal injections of the REF alone, in quantities used for incubation with normal serum, evoke no augmentation of erythropoiesis. Because of the relatively small quantities of REF employed, this may be the result of insufficient quantities entering the bloodstream, *via* the intraperitoneal route, to react with the serum component.

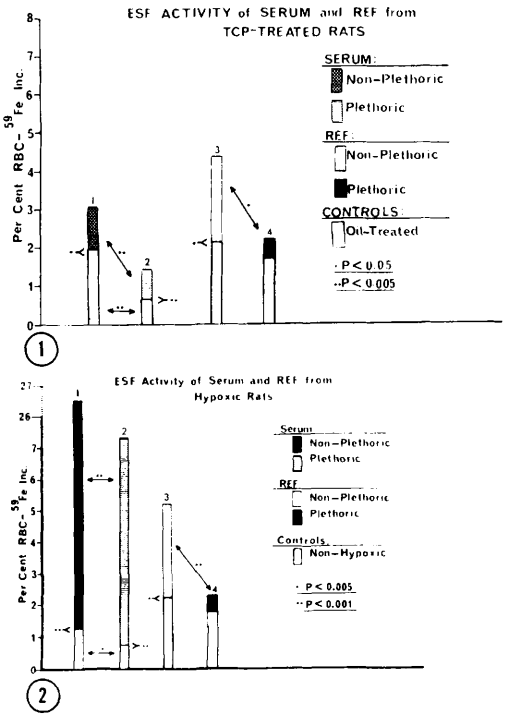

FIG. 1. Erythropoietin content of serum and renal erythropoietic factor activity in normal and plethoric rats given a single injection of testosterone cyclopentylpropionate and in cottonseed oil-treated controls.

FIG. 2. Erythropoietin content of serum and renal erythropoietic factor activity in normal and plethoric rats subjected to 0.4 atm. for 16 hr and in unexposed controls.

Results. Androgen groups. Fig. 1, Bar 1 indicates that the sera of normal, non-plethoric rats given a single injection of TCP showed significantly greater erythropoiesis-stimulating activity than the vehicle-treated group. The sera obtained from the cottonseed oil-injected controls appeared, in this experiment, to stimulate slightly RBC-radioiron incorporation in assay mice. However, this effect has not been consistently observed. TCP also resulted in a small but statistically significant increase in ESF content of the sera of plethoric rats (Fig. 1, Bar 2). The quantity present in the sera of plethoric rats, however, was less than in non-plethoric rats receiving the same dose of TCP. Bar 3 shows that TCP caused a considerable increase in the REF activity over that of the oil-injected

controls. In this regard, kidneys from normal untreated rats have been demonstrated to contain small but significant quantities of the REF(13,14). A small but statistically insignificant increase in REF activity was noted in the androgen-treated plethoric rats (Bar 4). The degree of activity here was considerably less than that noted in the non-plethoric group.

Hypoxia group. A highly significant increase in ESF titers in the serum occurred in normal rats exposed to hypoxia (Fig. 2, Bar 1). Although a rise in serum ESF concentration was also observed in hypoxia-exposed plethoric rats (Bar 2), it was much less than that noted in the non-plethoric group (*i.e.,* in the latter animals, 1.0 ml of serum produced a 26.5% RBC-radioiron incorporation as against a 7.6% value in plethoric rats). Similar results were obtained for the REF. Whereas a significant increase in REF activity was found in the hypoxia-exposed non-plethoric animals (Bar 3), a small, statistically insignificant rise was seen for the REF in the plethoric rats (Bar 4) subjected to hypoxia.

Discussion. The present work supports and extends previous observations that one of the mechanisms by which androgen augments erythropoiesis is through an increase in the quantities of circulating ESF(9,10). The erythropoiesis-stimulating activity of serum from androgen-treated animals is not attributable to residual androgen. This is seen from experiments indicating that: 1. the stimulatory effects of androgen on erythropoiesis wane with continued treatment(8,9); 2. large doses of testosterone are less effective than moderate doses(17); 3. plasma from androgen-treated animals, in amounts which stimulate erythropoiesis, fails to increase reproductive accessory weights in immature mice(17). Moreover, the contention that androgen operates by evoking the production of ESF is strongly supported by the finding that the stimulation of erythropoiesis produced by testosterone does not occur in the presence of an immune serum against ESF(18,19).

That a renal mechanism mediates the effects of testosterone on erythropoiesis is seen from the fact that this steroid fails to produce its stimulatory effects on erythropoiesis in nephrectomized mice(19). Likewise, whereas androgen augments the erythropoietic response to hypoxia in intact mice, it is incapable of producing this action in mice binephrectomized just prior to exposure to hypoxia(20). Moreover, perfusion of kidneys from previously androgenized rabbits with normal blood results in continued production of ESF over a 3½ hour perfusion period(17).

The data further indicate that the rise in serum ESF caused by androgen or hypoxia is positively correlated to the increase in REF activity evoked by these 2 stimuli in normal rats. We have previously shown that similarly prepared "light mitochondrial" extracts of liver are ineffective in producing ESF when incubated with normal serum(14). A point of interest emerging from the present study is that hypertransfused rats produce considerably less ESF in response to androgen or hypoxia than normals and this appears to be a direct consequence of an impaired production of the REF in the plethoric animal.

The mechanism by which plethora acts to suppress the production or activation of the REF remains conjectural. Its relation to the possible presence of an inhibitor in the blood of plethoric animals(21,22) requires exploration. It might also be attributable to an increased oxygen supply to the kidney associated with the greater numbers of circulating red cells or it might be caused by some as yet imperfectly understood influence of the higher blood viscosity associated with the plethoric state(23,24).

Summary. The appearance of increased quantities of ESF in the sera of rats following androgen administration or exposure to hypoxia has been related to increased amounts or activity of the renal erythropoietic factor (REF). Plethora induced in rats by hypertransfusion results in a marked decrease in REF activity in response to androgen or hypoxia. This is proposed as the mechanism by which plethora inhibits the production of ESF.

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Bilirubinemia in the Polycythemia of High Altitude.* (31519)

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Hurtado *et al*(1) and Merino(2) have pointed out that approximately half of the Andean natives, living at high altitude and in apparent good health, show an increase in the amount of plasma bilirubin mainly due to an elevation in the indirect fraction (not conjugated) of the pigment. The causes for the elevation of indirect bilirubin are not known. However, the following probable mechanisms have been suggested:

A) an increase in total amount of daily red blood cell destruction paralleling the elevation of red blood cell mass; and

B) a mild reversible "hepatic insufficiency," probably due to the hypoxia.

No real proof has been presented to support either mechanism.

Since conjugation of bilirubin seems to be essential for its excretion through the bile, a defect in glucuronide production could cause an alteration in the pigment excretion with consequent retention and in increase in the plasma of unconjugated bilirubin(3).

Some authors(4,5,6) have used substances such as salicylamide and menthol to test the ability of glucuronide production by subjects showing an indirect hyperbilirubinemia without signs of over hemolysis, measuring its excretion in the urine as a glucuronide.

The present study was undertaken to determine whether some alteration in glucuronide conjugation takes place in natives living at high altitude.

Material and methods. Eleven men in good health were chosen as the control group for the studies done at sea level. All had lived in Lima (150 meters above sea level) for many years; none had been subjected to low oxygen barometric pressure for several months prior to this study. Nineteen men in good health were chosen as members of the high altitude group; all had been residents of Cerro de Pasco (4,390 meters above sea level) for many years.

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