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Inhibition of Erythropoietic Effects of Hormones by Erythropoietin Antisera in Mildly Plethoric Mice.* (32375)

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Hormonal agents from the pituitary(1,2,3), thyroid(4,5,6,7), adrenal(8,9) and testis(3,6, 8,10,11,12) have been reported to stimulate erythropoiesis in the endocrine deficient as well as the normal animal. However, the mechanism by which these humoral substances stimulate erythropoiesis is not clearly understood. In a previous study(3) we demonstrated that the erythropoietic effects of ACTH, thyroid hormones and adrenocortical steroids in hypophysectomized rats were associated with an increase in oxygen consumption. Shalet *et al*(7) recently reported that 3,5,3′-triiodothyronine (T_3) did not stimulate erythropoiesis directly and suggested that T_3 exerts its effect by stimulating erythropoietin (ESF) production. Fried *et al*(13) and Gurney *et al*(6) have shown that plasma levels of ESF were elevated following testosterone injections and that mildly plethoric mice were more sensitive to the erythropoietic effects of androgens than polycythemic mice. Antiserum from rabbits immunized with ESF has also been found to block the action of ESF in polycythemic mice(14) and this effect is considered to be the result of an antigen-antibody combination. It has also been reported(15,16) that the erythropoietic effects of testosterone were completely abolished by ESF antisera. ACTH, 3,5,3′-triiodothyronine, and testosterone were studied in mildly plethoric mice in combination with erythropoietin antisera (Anti-ESF) in order to determine whether these hormones stimulate erythropoiesis by increasing erythropoietin production. The erythropoietic effects of these hormones were also compared in mildly plethoric and polycythemic mice.

Materials and methods. Mice were made polycythemic *via* a modification of the method of DeGowin *et al*(17). Male Swiss-Webster strain mice were injected with 1 mg of iron dextran prior to being placed at 0.45 atmosphere for 3 weeks. On the day of removal from the low pressure tank each mouse was injected intraperitoneally with 1.0 ml donor mouse blood at hematocrit 70. Either saline, sheep erythropoietin, or the humoral agents were injected subcutaneously into the mice each day for a period of 5 days following the transfusion. Each mouse was injected with 0.5 μc Fe^{59} i.p. on the 6th post-hypoxia day, exsanguinated on the 9th day and Fe^{59} incorporation in RBC determined.

The mildly plethoric mice were prepared according to the technique described by Fried *et al*(6). Female Swiss-Webster mice were injected i.p. with 0.2 ml donor mouse blood at hematocrit 80 on days 1 and 2 (total 0.4 ml) and injected with saline, sheep erythropoietin or the hormones each day for the first 5 days following transfusion. Fe^{59} was injected on the 6th day and 72 hour Fe^{59} incorporation in RBC determined on the 9th day as described above for the polycythemic mice. The hormones were injected subcutaneously and when they were administered at the same time as anti-ESF the injections were made at separate sites.

The erythropoietin antiserum was prepared by immunizing Race III strain albino rabbits with †Step III sheep erythropoietin

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† Step III sheep plasma erythropoietin from phenylhydrazine anemic sheep was supplied by USPHS Hematology Study Section.

according to the alum precipitate method of Kabat and Mayer(18). A total of 280 units of sheep erythropoietin was injected into 1 rabbit over a 5-week period. The total dose of sheep erythropoietin, alone or combined with anti-serum, was injected into polycythemic mice in 3 divided doses s.c. at 5-hour intervals on the 5th day following removal from the low pressure tank and transfused with 1.0 ml 70 hematocrit blood. Each mouse was injected with 0.5 μc Fe^{59} i.p. on the 7th post-hypoxia day and exsanguinated 3 days later for determination of Fe^{59} incorporation into red cells. The potency of the anti-ESF was such that 0.2 ml completely inhibited the response of polycythemic mice to 0.2 units sheep erythropoietin. The mean 72-hour Fe^{59} incorporation in RBC values of polycythemic mice (5 in each group) receiving 0.2 units ESF was 4.5 $\pm$ 1.94%; while mice receiving either saline or a combination of 0.2 units ESF and 0.2 ml Anti-ESF were .39 $\pm$ .04% and .41 $\pm$ .15% respectively. ACTH (Parke-Davis Corticotropin Injection) containing 5 mg aminoacetic acid per 40 units and standardized in terms of U.S.P. corticotropin standard was dissolved in 0.85% saline before injection. 3,5,3'-triiodothyronine (Smith, Kline and French Liothyronine Sodium) was dissolved in .01 N NaOH, placed in 0.85% NaCl solution and adjusted to pH 9.0 before injection. Testosterone propionate (Upjohn) in cottonseed oil was injected s.c. Plasma iron was determined on filtrates following precipitation with trichloroacetic acid with the use of a Model 303 Perkin-Elmer Atomic Absorption Spectrophotometer(19). The technique of analysis of variance(20) and the method of Dunnett(21) for comparing several treatments with a single control were used for the statistical analyses.

Results. As seen in Table I, sheep erythropoietin, testosterone and ACTH produced a slight but significant ($P<.05$) increase in Fe^{59} incorporation in RBC of polycythemic mice. 3,5,3'-triiodothyronine failed to produce a significant increase in Fe^{59} incorporation in polycythemic mice. On the other hand, erythropoietin, testosterone, 3,5,3'-triiodothyronine and ACTH produced a significant ($P<.01$) increase in Fe^{59} incorporation in RBC of mildly plethoric mice (Table II).

As shown in Table II, the erythropoietic effects of testosterone, 3,5,3'-triiodothyronine and ACTH in mildly plethoric mice were markedly enhanced when compared with the effects seen in polycythemic mice. Anti-ESF completely blocked the erythropoietic effects of these hormones in mildly plethoric mice. This inhibition is interpreted to be the result of an antigen-antibody combination. It is of interest to point out that the Fe^{59} incorporation values in all of the groups receiving Anti-ESF were significantly ($P<.01$) less than those of the saline controls. Thus providing evidence that the Anti-ESF cross reacts with and blocks mouse ESF. Therefore, the antagonism of the erythropoietic effect of the hormones in mildly plethoric mice by Anti-ESF can be assumed to be the result of neutralization of endogenous ESF. The mean plasma iron levels in the saline control mildly plethoric mice (382 $\pm$ 50.4 μg%) were higher than that of a group of 5 normal mice (285 $\pm$ 32.4 μg%). However, the plasma iron values in the mice receiving testosterone, triiodothyronine or ACTH were not significantly different from that of the mildly

TABLE I. Influence of Hormones on Radioactive Iron Incorporation in RBC of Polycythemic Mice.

Treatment	Dose* (per day)	No. mice	Hematocrit (%)	% Fe^{59} incorp. RBC
Saline	—	9	68 $\pm$ 1.66	1.04 $\pm$ .30
Erythropoietin	.2 units	9	67 $\pm$ 2.02	6.79 $\pm$1.21†
Testosterone	2.5 mg	6	66 $\pm$ 2.21	8.21 $\pm$3.27†
3,5,3'-Triiodothyronine	5 μg	6	60 $\pm$ 1.44	.94 $\pm$ .23
ACTH	2.5 units	6	64 $\pm$ 2.51	2.17 $\pm$ .62

$\pm$ = Standard error of mean.
* Each mouse injected s.c. with daily dose indicated for 5 days.
† Significantly different from saline controls at the 5% level.

TABLE II. Effects of Several Hormones Alone or in Combination with Anti-ESF on Fe^{59} Incorporation in RBC of Mildly Plethoric Mice.

Treatment	Dose* (per day)	No. mice	Hematocrit (%)	Plasma iron (μg/100 ml)	% Fe^{59} incorporation
Saline	—	14	46.7 ± 1.19	382 ± 50.4	7.85 ± 1.84
Erythropoietin	.2 units	19	43.6 ± .92	443 ± 22.3	22.84 ± 2.25†
Testosterone	2.5 mg	11	45.3 ± .84	383 ± 25.1	27.44 ± 4.9 †
" + anti-ESF	2.5 mg	7	45 ± 2.22		1.87 ± .33
3,5,3′-Triiodothyronine	5 μg	14	45.2 ± .76	278 ± 17.4	28.72 ± 2.55†
" + anti-ESF	5 μg	8	43 ± 1.98		.56 ± .06
ACTH	2.5 units	13	44.5 ± 1.13	410 ± 38.0	18.62 ± 2.87†
" + anti-ESF	2.5 units	8	40 ± 2.87		1.40 ± .34

* Each mouse injected s.c. for 5 days.

† Mean value is significantly ($P < .01$) greater than that of the saline controls. All groups receiving anti-E plus hormone were significantly ($P < .01$) less than their respective groups receiving hormone alone.

Mean hematocrit for normal mice(16) was 40 ± .62%.

plethoric saline controls. Therefore, it seems unlikely that the increase in Fe^{59} incorporation seen in mildly plethoric mice injected with hormones could have been due to a decrease in plasma iron.

Discussion. The present findings that Anti-ESF blocks the erythropoietic effects of 3,5,3′-triiodothyronine, ACTH, and testosterone in mildly plethoric mice indicates that the primary mechanism by which these hormones augment erythropoiesis is either through an increase in endogenous production of erythropoietin or that ESF is required for these substances to stimulate erythropoiesis. An inhibitory effect of Anti-ESF on the erythropoietic action of testosterone as well as the increased sensitivity of mildly plethoric mice to both T_3 and testosterone, when compared with polycythemic mice, has been reported previously(6,15,16). However, this is the first report that the effects of ACTH on erythropoiesis are markedly enhanced by mild plethora and that the erythropoietic effects of 3,5,3′-triiodothyronine and ACTH are blocked by antisera to ESF. The mechanism by which increasing degrees of plethora dampens the erythropoietic effects of these hormones is not known. Other investigators(6,7) have attributed the progressive loss of the erythropoietic effect of testosterone and T_3 with increasing plethora to a reduction in the tissue oxygen demand-supply ratio. The oxygen supply is probably increased by polycythemia to such an extent that the hypoxic effects attending the enhanced cellular utilization of oxygen, produced by the hormones, overwhelms the tissues by the excessive oxygen supply available. Recently transfusion plethora was also found to markedly reduce a renal erythropoietic factor postulated to be involved in a step in the production of ESF (12). The possible role of inhibitors in plasma(22,23) and/or kidneys(24) of plethoric animals requires further study.

The kidney is known to be the principal site of ESF production. Therefore, the effects of T_3, testosterone and ACTH on ESF production are probably mediated through the kidney. Testosterone(16) and T_3(7) fail to produce their stimulatory effects on erythropoiesis in nephrectomized animals. The rise in serum ESF seen in testosterone-treated intact animals has also been correlated with an increase in a renal erythropoietic factor (12). ACTH exerts its primary effects on ESF production secondarily through the release of adrenocortical steroids(2). However, ACTH has also been found to stimulate erythropoiesis in hypophysectomized-adrenalectomized rats(25). Activation of a renal enzyme through a cellular hypoxic mechanism is the most plausible explanation for the stimulatory effects of the hormones on ESF production in the present study.

Summary. Antisera to erythropoietin (Anti-ESF) was found to block completely the erythropoietic effects of 3,5,3′-triiodothyronine (T_3), ACTH, and testosterone in mildly plethoric mice. The effects of T_3, ACTH, and testosterone were also markedly dampened by increased plethora. The hormones studied increased radioactive iron in

red cells of mildly plethoric mice without producing a significant alteration in plasma iron. These findings indicate that ACTH, testosterone and T_3 stimulate erythropoiesis indirectly by increasing the elaboration of endogenous erythropoietin, or else they require a certain level of erythropoietin in the recipient animal for their erythropoietic effects.

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Relationship Among Nursing Frequency, Lactation Pituitary Prolactin, and Adrenocorticotropic Hormone Content in Rats.* (32376)

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Maintenance of mammary structure and synthesis during lactation is dependent on frequent nursing(1). Adjusting litter size to higher numbers caused significant progressive increases in mammary deoxyribonucleic acid (DNA) totaling more than 100% during the first 16 days of lactation, whereas low nursing intensities did not significantly change mammary DNA content. Greater nursing intensities correspondingly increased ribonucleic acid (RNA) and RNA/DNA values, which suggested that the mammary cells produced during lactation were functional.

Short-term suckling following a 12-hour non-nursing period caused prolactin release (2,3) which was not fully restored to pre-

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