# Effective Erythropoiesis Induced by 5β-Pregnane-3β-Hydroxy-20-One in Squirrel Monkeys

EMMANUEL C. BESA, DOV GORSHEIN, WILLIAM A. HAIT, and FRANK H. GARDNER

*From the Hematology Research Laboratory, Presbyterian-University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104*

ABSTRACT The erythropoietic effect of 5β-pregnane-3β-hydroxy-20-one, a naturally occurring steroid metabolite of progesterone, was evaluated in the squirrel monkey by ferrokinetic studies, red cell survival, and blood volume measurements. The intramuscular administration of this steroid in pharmacologic doses shortened the $^{59}$Fe plasma clearance and increased the plasma iron turnover, thereby indicating an increase in erythropoiesis. A normal $^{59}$Fe red cell uptake was observed, and the bone marrow maturation time was not altered. Red cell survival was the same in the treated and control groups. After five weekly injections of the steroid, the monkeys increased their red cell mass by 57%. A significant increase in white blood cells and a slight elevation of platelet counts in the treated monkeys also suggest a possible direct stimulation of hemopoietic stem cells by the steroid metabolite.

These observations indicate that some steroid metabolites can stimulate an early increase in iron turnover (within 48 h) that is not secondary to hemolysis. The increased red cell mass indicates effective erythropoiesis in primates.

## INTRODUCTION

Recent data regarding the metabolism of androgens suggest that testosterone is transformed into different active metabolites for use by the target organs (1, 2). These metabolites may have different effects, and currently the summation of the different metabolite functions is used to define the action of the parent compound. Thus the anabolic, masculinizing, and erythropoietic activity of androgens could very well be a summation of different activities of various metabolites. The need for a hematopoietic agent void of virilizing and salt-retaining properties led us to search for an active metabolite that is primarily erythropoietic.

The reduction of the Δ4 unsaturation of the A ring in testosterone results in the formation of 5α and 5β derivatives (1, 2). The 5α derivatives are androgenic but the 5β steroids were devoid of this property (3) in the rodent assay system. A group of naturally occurring metabolites with 5β-H configuration, in which the junction between the first two rings (A:B cis) of the steroid nucleus is highly angulated (Fig. 1), has been found to have a potent ability to induce heme synthesis (4) independent of erythropoietin (5, 6). The erythropoietic action of androgens in animals and man has been attributed to increase erythropoietin production (7, 8), with a possible direct bone marrow cell effect (9, 10). The stimulation of erythropoiesis by testosterone has been successfully blocked by antierythropoietin (8). The 5β-H steroids stimulate heme synthesis by increasing δ-aminolevulinic acid synthetase in the avian liver (11) and in blood islands of explanted de-embryonated chick blastoderm (12), and recently they have been reported to enhance heme and globin synthesis in human bone marrow culture (13–16). The 5β-H steroids were also capable of increasing $^{59}$Fe incorporation in red cells of normal and polycythemic mice and rats (5, 17). However, some investigators failed to demonstrate this effect in mildly plethoric (18) and polycythemic mice (19). The present in vivo study was undertaken to investigate the erythropoietic effects of a steroid metabolite in primates.

## METHODS

34 female and 4 male squirrel monkeys (*Saimiri sciureus*) 8–10 mo old with an average weight of 655 g, were individually caged and randomized equally into treated and control groups. The animals were given Rockland primate

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diet,[1] supplemented with fruits and water, ad libitum. 3 wk before the studies were begun all monkeys received 5 mg of iron-dextran (Imferon)[2] i.m. to ensure adequate iron stores. The cage areas were maintained on a 12-h on, 12-h off light cycle and at an average temperature of 25°C. Sodium pentothal (2 mg i.p.) was used to anesthetize the monkey. The femoral vein was always used to obtain blood or for intravenous administration. Complete blood counts were done using standard techniques. All radioactive counts were done in an automatic well-type scintillation counter.[3]

The treated group (5β-H group) was given 5β-pregnane-3β-hydroxy-20-one[4] (Fig. 2), dissolved in 10% *N,N*-dimethylacetamide and propylene glycol (1,2-propanediol)[5] at 25 mg/kg body weight i.m. (0.2 ml); the control group received the diluent (vehicle) only. Dosage schedules were as follows: for the ferrokinetic studies, steriod or vehicle was injected 48–72 h before measurements were done. For the red cell survival and blood volume studies, the monkeys were given five weekly injections of the steroid or the vehicle.

*Ferrokinetics.* Plasma iron clearance, plasma iron turnover, $^{59}$Fe red cell incorporation, and marrow maturation time were determined using methods previously described (20, 21) with the following modifications for microtechnique. The femoral vein was cannulated with an intramedic (PE-90/S12′) polyethylene catheter[6] for repeated removal and readministration of blood. Initially, 6 ml of blood were removed for plasma iron determination. From this sample, 1.5 ml of plasma were incubated with 0.25 μCi of $^{59}$Fe citrate[7] at 37°C for 20 min, and 1 ml of the labeled plasma was injected i.v. Thereafter, 50 μl of plasma were separated from 1 ml of blood drawn at 5, 15, 30, 60, 90, 120, and 180 min. The radioactive counts were obtained from all samples and plotted on semilogarithm paper to calculate the plasma iron clearance. After reconstitution, all red cells were reinfused with saline to maintain the monkey's hematocrit. All studies were initiated at 2:00 p.m. each day to minimize the diurnal variation in plasma iron. Plasma volume was calculated by $^{59}$Fe dilution at 0 time.

*$^{59}$Fe red cell uptake and marrow maturation time.* $^{59}$Fe red cell uptake was determined and completed in 10 monkeys. The same procedure was done as for the clearance studies and continued by counting 100 μl of blood radioactivity at days 1–5, 7, 9, 11, and 15. Results were plotted as percent of $^{59}$Fe uptake.

*Blood volume determinations.* A modification of the method of Read (22) to measure blood volume was used for microtechnique before the first administration of the vehicle or the steroid, and a week after the last injection. A sample of 2 ml of blood was drawn and immediately placed in a sterile vial with 0.25 ml acid citrate dextrose[7] (special formula). Sodium-51chromate[8] (5 μCi) was added and incubated at 37°C for 45 min, and 5 mg of ascorbic acid was added. For the whole blood standard sample, 0.5 ml of blood was removed, diluted to 1.0 ml, and from this mixture 0.1 ml was re-injected. The hematocrit was measured after the dilution. Radioactive counts were determined from the rest of the sample by pipetting 100 μl whole blood and 50 μl of plasma standard into a well-type scintillation counter. The same amount of whole blood and plasma was counted from the 30-min sample, and the hematocrit was measured. Calculations of the red cell mass were done with the standard formula correcting all volumes of 1 ml as follows:

$$RCM = \frac{[(\text{CWB Std})(10)] - [(1 - \text{Hct WB Std}) \times (\text{CPl Std})(20)] \times 30' \text{ Hct}}{\text{C30' WB} - [(1 - 30' \text{ Hct})(\text{C30' Pl})]}$$

Where *RCM* = red cell mass, CWB Std, CPl Std, C30′ WB, C30′ Pl are cpm of whole blood standard, plasma standard, 30′ whole blood or 30′ plasma sample, respectively. Plasma volume was calculated from the red cell mass and venous hematocrit.

*Red cell survival.* Red cell half-life was measured after the second blood volume determination using the $^{51}$Cr-tagged red cells given for the volume measurements. 1 ml of blood was drawn at 24 h and weekly thereafter for 21 days, and 100-μl aliquots were counted for radioactivity. Counts were plotted on semilogarithm paper, and the erythrocyte half-life was calculated. The 30-min whole blood was used as the 100% baseline value.

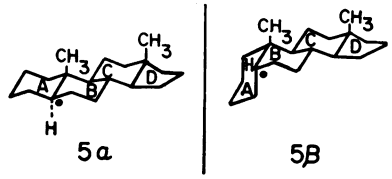


FIGURE 1 Sterochemical representation of 5α-H and 5β-H isomeric forms of the steroid nucleus from Levere, Gordon, Zanjani, and Kappas (4).

## RESULTS

*Ferrokinetics.* The plasma iron levels in the control and the 5β-H groups are noted in Table I. The mean plasma volume, as determined indirectly by the $^{51}$Cr dilution technique, was 17.9 ml, and 19.1 ml for the 5β-H group and the control group. The pretreatment $^{59}$Fe dilution during the procedure gave plasma volumes of 18.8 ml and 20.1 ml, respectively, showing no significant

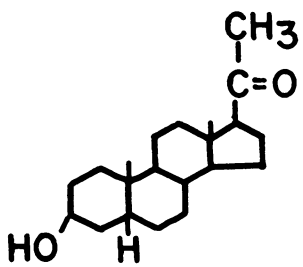


FIGURE 2 Structural formula of 5β-pregnane-3β-hydroxy-20-one.

[1] Tekland, Inc., Monmouth, Ill.
[2] Lakeside Laboratories, Inc., Milwaukee, Wis.
[3] Baird Atomic, Inc., Bedford, Mass.
[4] Kindly provided by Dr. John C. Babcock of The Upjohn Co., Kalamazoo, Mich.
[5] Fisher Scientific Co., Pittsburgh, Pa.
[6] Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.
[7] Abbott Scientific Products Div., Abbott Laboratories, South Pasadena, Calif.
[8] Cambridge Nuclear Radiopharmaceuticals, Cambridge, Mass.

TABLE I

*Ferrokinetic Studies in Groups of Six Monkeys Treated with 5β-H Steroid and Controls*

| Experimental group | Weight | Plasma iron clearance | Plasma iron | Plasma iron turnover | 80% $^{59}$Fe uptake | Marrow maturation time |
|---|---|---|---|---|---|---|
| | *g* | *min* | *μg/100 ml* | *mg/24 h* | *days* | *days* |
| Control | 660±5 | 102±7.6 | 137±11 | 0.28±0.03 | 5.6±0.3 | 2.3±0.3 |
| Steroid (5β-H) | 670±4 | 71±1.5 | 162±28 | 0.51±0.1 | 4.9±0.9 | 2.2±0.3 |
| *P* value | NS | <0.005 | NS | <0.02 | NS | NS |

Values expressed as mean±SEM.

change in the volume, thus excluding false clearance values. The average of the $^{59}$Fe counts per 10 min at start of the study was 57±8.0, and 15±9.2 at the end of 180 min in the 50-μl aliquot of plasma. The mean plasma iron turnover and plasma iron clearance for the control and the 5β-H groups are listed in Table I. Fig. 3 depicts a significantly shortened ($P < 0.005$) plasma iron clearance and a twofold increase ($P < 0.02$) in plasma iron turnover after treatment with the metabolite.

$^{59}$Fe red cell uptake was 80% to 100% at 7 days for the control group and the 5β-H group, except for two monkeys with 80% at 3 days. However, the mean marrow maturation time was the same for both groups as listed in Table I.

*Blood volumes.* The red cell mass ($^{51}$Cr) of a nontreated 655-g monkey averaged 13.3±0.7 ml (pretreatment values for both treated and control monkeys). After treatment for 5 wk, the red cell mass of the control group did not change, while the 5β-H group increased their red cell mass (Table II). Plasma volume in the 5β-H group increased 10.6 ml ($P < 0.001$).

*Red cell survival.* Data for both groups are listed in Table II and show no significant difference between the 5β-H and the control groups. The half-life of 15 days as measured by $^{51}$Cr has been observed in the small primates by other workers using the same technique (23).

*Blood counts.* The white cell counts in the 5β-H and control groups after 5 wk of drug administration were 10,400 mm$^3$ and 6,700 mm$^3$/μl, respectively, ($P < 0.01$) both with normal differential. The 5β-H group had a slight elevation ($P < 0.2$) of the mean platelet count to 350,000 mm$^3$/μl (control = 295,000 mm$^3$/μl). The hematocrits of both groups did not change compared to their pretreatment levels. The 5β-H group maintained its hematocrit by elevating both the red cell mass and the plasma volume.

## DISCUSSION

A number of steroid metabolites with the 5β-H configuration has been known to play a role in regulation of erythropoiesis. The increase in heme-prophyrin biosynthesis in the erythroid models was related to the induction of δ-aminolevulinic acid synthetase (12), the rate-limiting enzyme in the production of heme (24). It is of interest that erythropoietin was shown to in-

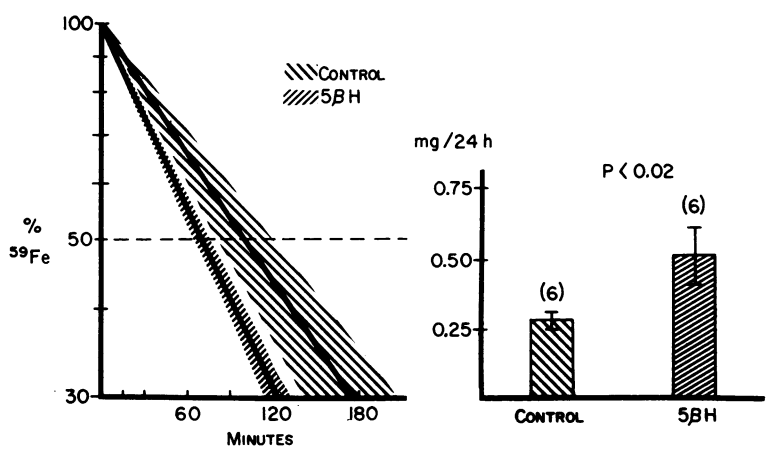


FIGURE 3 Ferrokinetic studies showing a short plasma iron clearance (left) and twofold increase in 24-h plasma iron turnover (right) in the steroid-treated monkeys as compared to control. Numbers in parentheses indicate monkeys in each group.

TABLE II

*Red Cell Mass and Red Cell Survival Data*

| Experimental group | No. | Red cell mass wk 0 | | Red cell mass wk 5 | Change | Red cell half-life |
|---|---|---|---|---|---|---|
| | | *ml* | | *ml* | % | *days* |
| Control | 17 | 13.7±0.4 | (NS) | 13.7±0.4 | 0 | 13.7±0.7 |
| Steroid (5β-H) | 16 | 12.3±0.4 | ($P < 0.001$) | 19.1±0.8 | +57 | 14.6±1.4 |

Values expressed as mean±SEM.

crease δ-aminolevulinic acid synthetase in the rabbit bone marrow culture (25), suggesting a similarity in the site of action between the 5β-H steroids and erythropoietin. However, plasma from 5β-H steroid-treated rats (5) and mice (6) failed to demonstrate any erythropoietic activity. The erythropoietic activity of the steroid could not be abolished by antierythropoietin (5). These observations may imply that the 5β-H steroid metabolite has a unique role in the regulation of red cell production that is not related to erythropoietin. Byron (26, 27) and other workers (28) demonstrated another role of these steroids. The 5β-H steroid triggers the hemopoietic cells into cycle, presumably "pushing" stem cells into an erythropoietin-sensitive state (29). However, this "cycling" effect on hemopoietic stem cells was also described with dibutyryl cyclic AMP (26) and testosterone (27) and thus is not unique to the 5β-H steroid. Necheles observed a synergistic effect with erythropoietin in stimulating $^{59}$Fe uptake in human marrow cultures if the 5β-H steroid was added 24 h before erythropoietin. He thus proposed that induction of δ-aminolevulinic acid synthetase plays a role in the regulation of the stem cell differentiation into the red cell series (30).

In the current study we found shortening of plasma iron clearance and an increased plasma iron turnover 48–72 h after the 5β-H steroid was administered. One possible interpretation is that induction of δ-aminolevulinic acid synthetase and thus differentiation of the stem cell (30), induced by the 5β-H steroid, took place within 24 h. These triggered cells became receptive to erythropoietin, thereby differentiating into erythroid cells. The erythropoietic response, in turn, is reflected 48 h later by a rapid iron clearance and an increased iron turnover. This time sequence is compatible with the previous observations about erythropoietin cell responsiveness (30). The acceleration of the plasma iron clearance induced by the steroid may reflect an increased marrow response with greater production of hemoglobin (31). Plasma iron clearance is affected by several other conditions, such as ineffective erythropoiesis or hemolysis (31). The red cell survival studies suggest a normal erythrocyte life-span in the steroid-treated monkeys, thus eliminating the possibility of hemolysis, and the presence of short-lived cells of stress erythropoiesis (32). The increase in red cell mass indicates that this is in fact an effective erythropoiesis.

The increase in platelets and leukocyte count suggests not only an erythroid stimulation but also possibly a direct stem cell effect. These increments, which occurred in the presence of an expanded blood volume, are more significant in view of the possible dilution factor. This stem cell differentiation from other cell lines may be more prominent with the 5β-H steroids as compared to androgens, possibly because of the absence of endogenous erythropoietin stimulation with the steroid metabolites. A possible shift between circulating and marginal granulocyte pool may also explain a high leukocyte count. This so-called pseudoneutrophilia can occur acutely after administration of epinephrine or etiocholanolone (33). However, in this study neutrophilia was observed after 5 wk of steroid administration.

The increase in white blood count, platelets, and iron turnover could be partially explained by rapid release of cells from the bone marrow by the 5β-H steroid in addition to a stem cell stimulation. Recently, the 5β-H steroid has been reported to cause rapid release of reticulocytes from rat marrow (34). Although our 50% marrow maturation time was not significantly altered, two out of five monkeys in the 5β-H steroid group in our study incorporated more than 80% of the $^{59}$Fe in 3 days, as compared to a control group time of 7 days. The rate of release of other cell lines in this study was not measured.

The data thus presented, in conjunction with previous studies of the erythropoietic activity of the 5β-H steroids, demonstrate that these metabolites can not only stimulate activity via induction of δ-aminolevulinic acid synthetase in vitro in erythroid tissues of birds, rodents, and human bone marrow cultures, but also stimulate in vivo erythropoiesis in primates. The possible use of these naturally occurring metabolites of testosterone and progesterone should be evaluated in therapeutic programs for certain refractory anemias in man. The possible stimulatory effect on granulopoiesis and megakaryopoiesis

also suggests further investigation for possible use in disease states associated with pancytopenia and marrow suppression. The 5β-H steroids may be particularly useful as therapeutic agents in women and children, because they have been found to be devoid of the undesirable androgenic side effects in animal assay studies.

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