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Effects of Testosterone and 5β-Androstanes on *in Vitro* Erythroid Colony Formation in Mouse Bone Marrow[1] (41124)

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Abstract. A comparison was made of the effects of testosterone, 5β-dihydrotestosterone, and etiocholanediol on *in vitro* erythroid colony formation (CFU-E) in mouse bone marrow cultures. Dose–response curves with the three steroids in the presence of 0.2 unit Ep revealed that etiocholanediol at 10^{-11} – 10^{-7} *M* significantly enhanced CFU-E erythroid colony formation compared to 5β-DHT at the same doses. Testosterone at 10^{-8} *M* significantly enhanced colony formation compared to 5β-DHT at the same dose. It is postulated from these studies that the bone marrow may convert testosterone to 5β-DHT and the more erythropoietically active etiocholanediol which may be the active metabolite of testosterone stimulating *in vivo* erythropoiesis.

Current evidence suggests a structure–activity relationship for the action of androgenic steroids and their metabolites on erythropoiesis (1–7). Erythropoietin (Ep) production is enhanced in the perfused isolated dog kidney by testosterone and certain planar metabolites, such as 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one, 5α-DHT) (2). In contrast, 5β-H steroids have only slight but not statistically significant effects on Ep production (2). The effects of 5β-H steroids on erythropoiesis are somewhat controversial. Selected 5β pregnanes and androstanes have been shown to enhance radioiron incorporation in red cells of polycythemic mice (4, 5). On the other hand, a cooperative study of several steroid metabolites demonstrated very little effect on radioiron incorporation in red cells of exhypoxic polycythemic mice with the 5β-H steroids, whereas planar metabolites with the 5α-H configuration were very effective in stimulating radioiron incorporation into red cells of polycythemic mice (8). This *in vivo* effect in polycythemic mice could be partially mediated by increased erythropoietin production by the kidney and partially by direct action on the bone marrow. *In vitro* findings (1, 7), however, suggest that planar steroids are ineffective on bone marrow erythroid colony formation, whereas angular metabolites such as 5β-dihydrotestosterone (17β-hydroxy-5β-androstan-3-one, 5β-DHT) are effective in this system.

The finding that testosterone is not converted to 5α-dihydrotestosterone by bone marrow cells suggests that the metabolism of androgens may be different in this tissue as compared to the kidney or prostate which have 5α-reductase activity and thus are capable of this conversion (9). Studies by Levere *et al.* (10) in the chick blastoderm demonstrated that hemoglobin synthesis was markedly stimulated by certain 5β-androstane steroids such as etiocholanolone (3α-hydroxy-5β-androstan 17-one) and etiocholanediol (5β-androstane-3α, 17β-diol). The purpose of the present study was to characterize the effect of etiocholanediol, a naturally occurring 5β-metabolite of testosterone, on erythroid colony formation in mouse marrow cells.

Methods. Female mice (CD-1) weighing 25–30 g were obtained from Charles River Laboratories. Prior to the *in vitro* experiments mice were sacrificed by cervical dislocation and the femurs were removed and flushed with cold sterile alpha medium (11) (Flow Labs, Rockville, Md.) contain-

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ing 2% fetal bovine serum. Penicillin (200 units/ml) and streptomycin (200 μg/ml) were included to prevent bacterial growth. After flushing out the marrow contents a suspension of single cells was prepared by repeated passage through a 10-ml pipet and the cells were washed twice with cold collection medium. Nucleated cell counts were performed with Turk's solution and the cell suspension was adjusted to 5×10^6 cells/ml. Cells (5×10^5/ml) were plated in a mixture of alpha medium containing 0.8% methyl cellulose (Fisher Scientific Co.), 23% fetal bovine serum (Flow), penicillin (200 units), streptomycin (200 μg), 0.5 m*M* mercaptoethanol, 0.2 unit of human urinary erythropoietin[3] and varying concentrations of 17β-hydroxy-4-androsten-3-one (testosterone) 5β-DHT, or etiocholanediol. Each steroid was dissolved in absolute ethanol prior to addition to the culture. One milliliter of methyl cellulose mixture was plated per petri dish (10 × 35 mm) in duplicate. Cultures were incubated for 2 days at 37° in a humidified atmosphere of 95% air and 5% CO_2.

Colonies were observed after staining for red cell pseudoperoxidase activity by incubating with the substance 3,3′-diamino benzidine (DAB) (12). Three milligrams of DAB were dissolved in 10 ml of 0.05 *M* Tris−HCl buffer, pH 7.6. Two milliliters of 3% hydrogen peroxide solution were added to the DAB solution just prior to use and approximately 0.5 ml of the mixture was layered over each petri dish from a Pasteur pipet. Erythroid colonies appeared as brown clusters of eight or more cells and were counted in 1/16 of the plate area using a grid in the eyepiece of an Olympus inverted microscope at 100× magnification.

The mean number ± SEM of erythroid colonies was calculated from seven experiments. *P* values of less than 0.05 were considered significant as determined by Student's *t* test.

5β-Androstane-3α,17β-diol was prepared in the laboratory of one of us (A.S.) (Endocrine Research, Ochsner Medical Foundation, New Orleans, La.) by the reduction of 5β-DHT with sodium borohydride in ethanol. The melting point was 236−8° (Lit. (13) mp, 236−236.5°).

Results. The chemical structures and spatial configurations of testosterone, 5β-dihydrotestosterone and etiocholanediol are shown in Fig. 1 (14). Etiocholanediol differs from 5β-DHT in having a 3α-hydroxy configuration in the A ring instead of the 3-keto group as seen with 5β-DHT. Both 5β-steroids are essentially nonandrogenic as compared to testosterone which is highly androgenic.

The three steroids were compared in a dosage range of $10^{-11}-10^{-7}$ in the presence of 0.2 unit erythropoietin. As seen in Fig. 2, etiocholanediol was more effective than testosterone or 5β-DHT in producing an increase in erythroid colony formation. Etiocholanediol at 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} *M* was significantly ($P < 0.05$) greater than 5β-DHT at the same doses. Testosterone at 10^{-8} *M* significantly ($P < 0.05$) enhanced colony formation compared to 5β-DHT at the same dose. 5β-DHT was the least effective compound studied.

Discussion. The ability of etiocholanediol to enhance proliferation of CFU-E-type erythroid colonies further supports the hypothesis that structural alterations in the spatial configuration of the androgen, testosterone, confer specific effects on erythropoiesis while still reducing its androgenic effect. A highly angulated nucleus characteristic of 5β-H type steroids stimulates heme formation in suspensions of chick embryonic cells (15) or adult human bone marrow cells (7), whereas similar steroids with a planar nucleus (5α-H type) failed to exhibit this activity. Levere *et al.* (10) found that one of the most effective steroids on heme synthesis was etiocholanediol. Parsons (16) found that the intact chick blastoderms incubated with [4-^{14}C]testosterone at a time coincident with erythropoiesis resulted in the production of at least five metabolites, three of

[3] Human urinary erythropoietin for these studies was provided by the Department of Physiology, University of the Northeast, Corrientes, Argentina. This material was further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under USPHS Grant HE10880 (National Heart, Lung and Blood Institute).

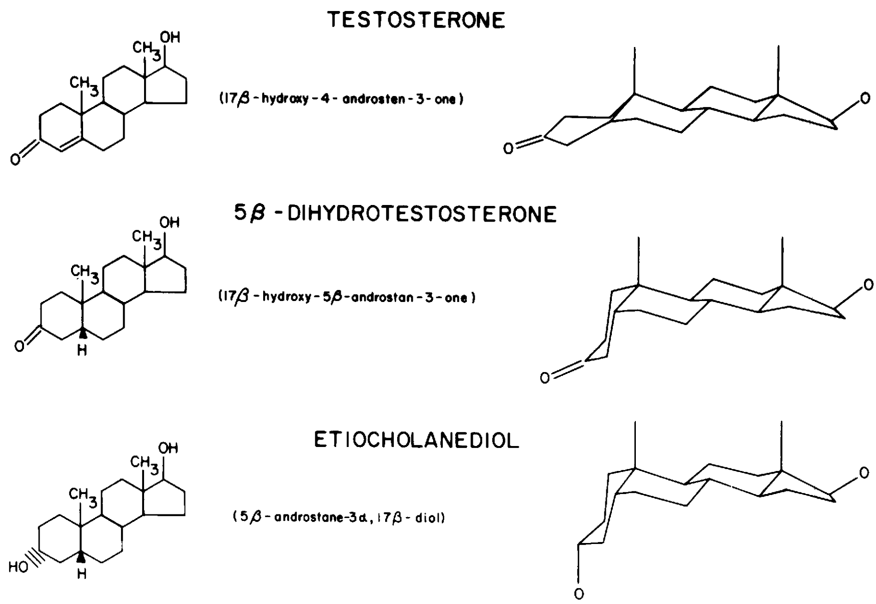

FIG. 1. Spatial configuration of the steroids studied.

which were 5β-androstane derivatives. Etiocholanediol accounted for 53% of the metabolites.

Our current hypothesis is that bone marrow is similar to the liver and less like the kidney in terms of response to steroids. Porphyrin biosynthesis in chick embryo liver cells is strongly stimulated by steroids with the 5β-H configuration. Etiocholanediol was reported (17) to have a potent effect on porphyrin synthesis and in man the liver contains a 5β-reductase capable of reducing 5β-H derivatives by an NADPH-requiring enzyme as well as 5α reduction. On the other hand, the kidney contains primarily 5α-reductase activity, and thus converts testosterone to 5α-DHT, an active metabolite in stimulating erythropoietin production; 5α-DHT is further converted in the kidney to 5α-androstane-3β,17β-diol and 5α-androstane-3α, 17β-diol. The fact that rat bone marrow apparently lacks 5α-reductase and thus cannot convert testosterone to 5α-DHT suggests the possibility that 5β-reductase may be present in bone marrow. 5α-DHT lacked any effect on erythroid colony formation in bone marrow cultures while 5β-DHT exerted a significant effect on erythroid cells (1). It is quite possible that the bone marrow may be converting testosterone to 5β-DHT and the more erythropoietically active etiocholanediol which could be the active steroid in the CFU-E compartment.

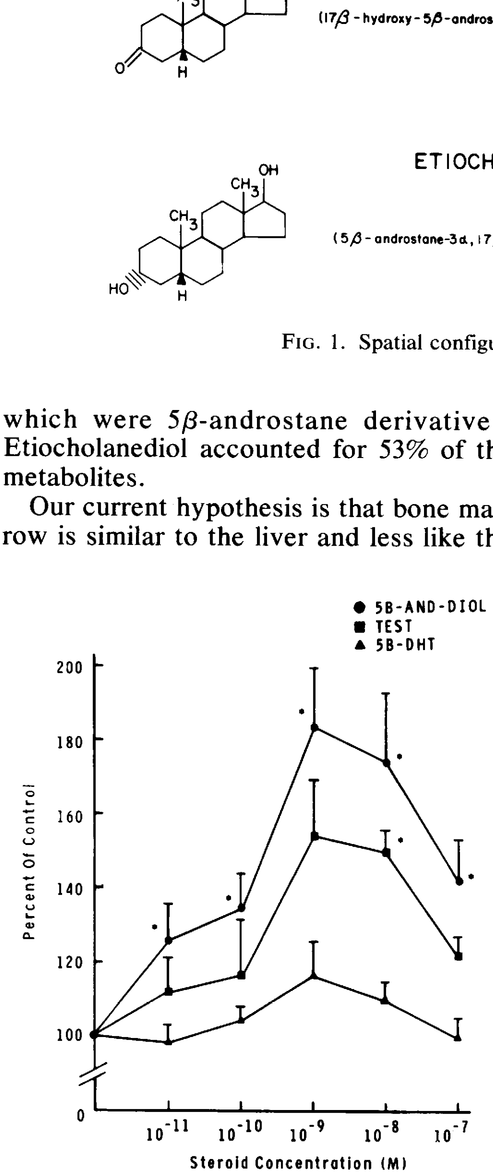

FIG. 2. Dose response to testosterone (■) 5β-DHT (▲), and etiocholanediol (5β-and-diol) (●) in the presence of 0.2 unit of Ep. *Significantly different ($P < 0.05$) from 5β-DHT at the same doses.

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