PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 178–180 (1975)

Increase in Erythroid Colony Formation in Rabbits Following the Administration of Testosterone[1] (38767)

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Testosterone was found to produce a significant increase in erythroid colony formation in rabbit bone marrow cultures *in vitro* using a methyl cellulose gel system (1). The increase in the number of erythroid colonies in cultures produced by testosterone *in vitro* was completely inhibited by the addition of busulfan (Myleran) to the culture medium (1). Busulfan has been postulated to block the formation of new erythropoietin-responsive cells (ERC) from hematopoietic stem cells (CFU) (2). Thus, these findings suggest that testosterone may act directly on CFU to enhance their differentiation into the ERC compartment and in the presence of erythropoietin (ESF) to cause an increase in nucleated erythroid cells (1, 3). It seems important in order to gain a better understanding of the mechanism of action of testosterone in erythropoiesis and in the treatment of patients with refractory anemias to determine whether the *in vivo* administration of testosterone increases erythroid colony formation similar to that seen following *in vitro* bone marrow stimulation with testosterone (1, 3).

Therefore, the present study was undertaken primarily to determine whether testosterone injections *in vivo* increases erythroid colony formation *in vitro* in bone marrow cultures.

Materials and Methods. Young female New Zealand rabbits weighing approximately 2.5 kg were treated as follows: *Group I* served as controls (no busulfan or testosterone treatment); *Group II* animals were injected with testosterone (5 mg/kg) im and then killed for the bone marrow cultures 18 h later; and *Group III* rabbits were given busulfan (20 mg/kg) orally and followed 24 h later by testosterone propionate (5 mg/kg) im and sacrificed for cultures of erythroid colonies 18 h after the injection of testosterone; *Group IV* rabbits were given busulfan (20 mg/kg) orally and sacrificed for cultures of erythroid colonies 42 h later.

A modification of the method of Iscove *et al.* (4) was used in the *in vitro* assay for erythroid colony formation as follows: Bone marrow cells were removed from the femurs of the rabbits treated as described above and flushed into alpha-medium, dispersed through a sterile pipette and washed twice with cold alpha-medium containing 10% fetal calf serum. For cultures of erythroid colonies, 2×10^5 nucleated marrow cells were plated in 35×10 mm plastic dishes in 1 ml alpha-medium containing 0.8% methyl cellulose, 30% fetal calf serum, 1% bovine serum albumin, penicillin (50 units) and streptomycin (20 μg). Three plates were prepared for each group. Cultures were incubated for 4 days at 37° in a humidified atmosphere of 5% CO_2 in air. Erythroid colonies containing 10 or more cells were scored on 0.25 of the total plate area using an inverted microscope at $75 \times$ magnification. Erythroid colonies were identified after staining with benzidine (5). For each experiment the number of erythroid colonies on each of three replicate plates was averaged and a mean and standard error determined.

Erythropoietin (ESF) levels of sera obtained from all three groups were assayed in the ex-hypoxic polycythemic mouse assay (6) to determine the level of endogenous ESF.

Results. Figure 1 shows the number of erythroid colonies formed in cultures from rabbits pretreated with either testosterone or busulfan plus testosterone. The number of erythroid colonies in bone marrows from rabbits (Group II) treated with testosterone *in vivo* was significantly ($P < 0.01$) higher than that of controls (Group I). However, this increase in the number of erythroid

[1] Supported by USPHS Contract No. AM 70-2112 from the Artificial Kidney/Chronic Uremia Program—NIAMDD.

colonies in cultures stimulated by testosterone *in vivo* was completely blocked by the concurrent administration of busulfan as seen in the rabbits (Group III) treated with busulfan orally and followed by testosterone. Note in Fig. 1 that busulfan treatment alone (Group IV) did not produce a significant effect on the ability of the rabbit bone marrow to form colonies *in vitro*. The number of colonies formed from bone marrows of the busulfan treated group were not significantly different from that of control marrows.

The effects of either testosterone alone or busulfan plus testosterone on endogenous ESF production are shown in Table I. Plasma ESF levels in each group were undetectable in our polycythemic mouse assay, indicating that the administration of either testosterone or busulfan plus testosterone did not affect erythroid colony formation in our culture system by increasing plasma ESF levels.

Discussion. The present studies indicate that erythroid colony formation in cultures is enhanced in bone marrows from rabbits pretreated with testosterone *in vivo* via a mechanism which is not erythropoietin dependent. This increase in erythroid colony-forming ability observed with testosterone treatment may result from either increased replication of ERC or from an increase in the differentiation of CFU into the committed erythropoietin responsive cell (ERC) pathway.

In an attempt to determine the mechanism of action of testosterone on erythropoiesis the effects of administration of busulfan before testosterone injections on erythroid colony formation were assessed. The increase in the numbers of erythroid colonies in cultures stimulated by testosterone *in vivo* was completely blocked when busulfan was given orally before injecting testosterone. Busulfan is postulated to block the formation of new ERC from CFU (2), thus indicating that testosterone probably acts directly on the CFU to induce differentiation of CFU into ERC. These findings were confirmed by our observation of an increase in erythroid colony forming ability in bone marrow cultures with the addition of testosterone *in vitro* (1). However, busulfan did not inhibit the increase in the numbers of erythroid

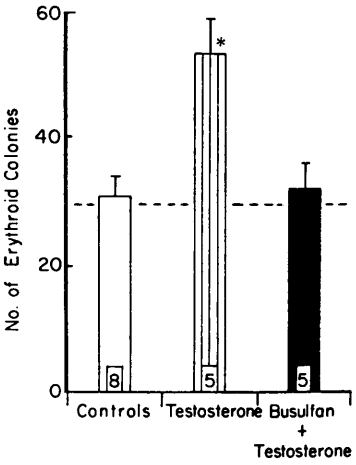

FIG. 1. Erythroid colonies formed in bone marrow cultures from rabbits pretreated with either testosterone alone or busulfan plus testosterone.

* Significantly ($P < 0.01$) different from both controls and busulfan + testosterone treated groups.

----- mean number of colonies in bone marrows from three different rabbits treated with busulfan alone.

TABLE I. SERUM LEVELS OF ERYTHROPOIETIN IN RABBITS TREATED WITH TESTOSTERONE AND BUSULFAN.

Treatment	No. of Experiments	ESF % ^{59}Fe incorporation into RBC ± SEM[b]
Saline		0.43 ± 0.14
0.05 Unit ESF		3.90 ± 0.72
Group I (controls)	(6)	0.70 ± 0.20[a]
Group II treated with testosterone alone	(5)	0.65 ± 0.12[a]
Group III treated with busulfan and testosterone	(5)	0.81 ± 0.22[a]

[a] Not significantly different from saline controls.

[b] Each mouse was injected s.c. with 1.0 ml of serum on the sixth and seventh day out of the hypobaric chamber.

colonies in cultures stimulated by ESF *in vitro*. This suggests that the mechanisms of action of testosterone and ESF on erythroid cell kinetics *in vitro* are different. Busulfan pretreatment of rabbits alone did not affect the ability of the bone marrow to form erythroid colonies in our culture system. Elson *et al.* (7) also reported that the rat

bone marrow only showed a slight decrease in cellularity 48 h after the administration of busulfan. The reason why busulfan inhibited the ability of testosterone to form erythroid colonies but did not affect the ability of ESF to form colonies can be explained by the fact that busulfan does not decrease the DNA content or DNA synthesis capacity of bone marrow cells (8). Therefore, it is possible that the new DNA synthesis required for cell division, which is not inhibited by busulfan, is an important site of action of ESF in enhancing erythroid colony formation. On the other hand, testosterone probably increases the numbers of erythroid colonies by inducing the CFU, which is sensitive to inhibition by busulfan, to differentiate into ERC. Thus, we postulate that testosterone acts on the CFU in a resting (G_o) stage (9, 10) to induce their differentiation into the ERC compartment causing an increase in the numbers of nucleated erythroid cells.

Summary. Changes in the numbers of erythroid colonies formed in cultures from rabbits pretreated with either testosterone or busulfan plus testosterone were studied using a methyl cellulose gel system. The mean number of erythroid colonies in bone marrows from rabbits treated with testosterone *in vivo* was significantly higher than that of controls. However, this increase in erythroid colonies in cultures seen following testosterone treatment was completely blocked by the concurrent administration of busulfan as seen in the rabbits treated with busulfan orally and followed by testosterone injections. Busulfan has been postulated to block the formation of new erythropoietin responsive cells (ERC) from hematopoietic stem cells (CFU). Thus, these findings suggest that testosterone may act directly on CFU to enhance their differentiation into the ERC compartment causing an increase in nucleated erythroid cells.

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Received October 10, 1974. P.S.E.B.M. 1975, Vol. 149.