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# Mechanisms of Action of Testosterone on Hemopoiesis *in vivo* and in Diffusion Chambers (40017)

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*Introduction.* It is now well established that androgenic hormones are capable of stimulating erythropoiesis in both experimental animals (1–3) and patients with various types of anemias (4, 5). However, the mechanisms by which these substances influence erythropoiesis remain unclear. It has been reported by several investigators that the erythropoietic effect of androgens is, in part, due to their ability to enhance the endogenous production of erythropoietin (ESF) (1.6–9). In other studies, the direct addition of androgens to *in vitro* marrow cultures has led to augmented erythroid differentiation. Using an *in vitro* methyl cellulose gel system, testosterone produced a significant increase in erythroid colony formation in both rabbit (10, 11) and human bone marrow cultures (12). In addition, in this system, a synergistic effect of a combination of testosterone and erythropoietin on erythroid colony formation was seen. This suggests that, in the presence of testosterone, a greater number of erythropoietin-responsive cells (ERC) is available for ESF to differentiate into the nucleated erythroid cell line.

Clinical experience with androgens in the therapy of aplastic anemia and of neutropenia induced by cytotoxic drugs suggests that these hormones may also stimulate granulopoiesis. The mechanisms by which androgens affect the granulocytic line are still largely unsettled. Rosenblum and Carbone (13) recently showed that *in vitro* addition of testosterone to cultures of human marrow increased the number of granulocyte colonies (CFU-C), and on cytokinetic grounds they attributed this effect to a direct stimulation of CFU-C proliferation.

In addition to these effects on more differentiated hemopoietic cells, testosterone may also have an effect on the pluripotential stem cell compartment. Byron (14, 15) found increasing sensitivity of pluripotential stem cells or spleen colony-forming units (CFU-S) to the lethal effect of high specific activity tritiated thymidine in testosterone-treated polycythemic mice. CFU-S of normal adult mice are generally insensitive to the cytocidal action of [$^3$H]thymidine. Hence the effect of testosterone was interpreted as either triggering CFU-S from resting ($G_0$) stage into cell cycle, or shortening the cell cycle of CFU-S.

The present study was undertaken to examine the granulopoietic effect of testosterone in mice, using an *in vivo* diffusion chamber (DC) culture system. Although *in vivo* studies are usually complicated by neutrophil egress from the marrow, cell-tight diffusion chambers allow for an assessment of total granulocytic differentiation.

To investigate the possibility of a direct action of testosterone on the morphologically unrecognizable progenitor cells, the effect on stem cell populations was also studied in intact mice. Both pluripotential spleen colony-forming cells (CFU-S) and recognizable granulocytic and erythroid cells were evaluated and compared to results in the diffusion chamber studies.

*Materials and methods.* Female $CF_1$ mice (20–25 g), 10–14 weeks of age, were used in all experiments.

Long-acting testosterone enanthate (Tstr) (Testoviron Depot, Shering Co.) was injected intramuscularly as an emulsion in sesame oil. Both intact animals and diffusion chamber-carrying mice received a single injection of 2.5 mg of long-acting testosterone on Day 0. Controls for both intact and chamber-carrying mice were injected with the same volume of the vehicle, sesame oil.

*In vivo studies.* At Days 0, 1, 3, and 7



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after treatment, a bone marrow cell suspension was obtained from the tibia of three to five mice by flushing out the marrow and drawing it back and forth through a 24-gauge needle. The total nucleated cellularity was determined by a Coulter counter Model B. Tibial marrow from at least three mice was pooled in sterile Hank's BSS with 100 units of potassium penicillin G and 100 $\mu$g of streptomycin sulfate per milliliter.

The suspension was then adjusted to 5 $\times$ $10^5$ cells/ml and 0.2 ml was injected into irradiated recipients to assess the number of marrow CFU-S by the spleen colony assay (16). Bone marrow smears for the differential counts were made from the contralateral tibia.

*Diffusion chamber studies.* The diffusion chamber (DC) technique as described by Benestad (17) was used. On Day 0, bone marrow cells were suspended in Hank's BSS containing 10% homologous serum, 100 U of potassium penicillin G, and 100 $\mu$g of streptomycin sulfate per milliliter.

The cells were counted in duplicate in a hemocytometer, and the cell suspension was adjusted with medium to obtain the final concentration. Each chamber was filled with 5 $\times$ $10^5$ cells/0.1 ml. Two chambers were implanted surgically into the peritoneal cavity of each recipient mouse under light ether anesthesia. Chambers were removed 1–7 days after implantation and shaken in a 0.5% Pronase and 5% Ficoll solution in Hank's BSS for 75 min, then cells were harvested by washing the chamber interior three times with Hank's BSS. The cell concentration per DC was determined by hemocytometer count. Slides were prepared with a Shandon cytocentrifuge and stained with May-Grünwald-Giemsa, and differential counts of at least 400 cells were made.

Cells were classified as immature proliferative erythroblasts (proerythroblasts and basophilic erythroblasts), mature erythroblasts (polychromatophilic and orthochromatic erythroblasts), proliferative granulocyte precursors (myeloblasts, promyelocytes, and myelocytes), mature nonproliferative granulocytes (metamyelocytes and granulocytes), macrophages, lymphocytes, and other cells (megakaryocytes, unrecognizable, and nonhemopoietic cells).

The number of CFU-S per DC was determined as follows. Contents from six to eight DCs were pooled and 1 $\times$ $10^5$ cells were injected into groups of seven to nine mice previously given 950-R total-body irradiation with an X-ray apparatus under the following conditions: 200 kV, 20-mA filtration, 0.5 mm Al + 0.5 mm Cu, 70 rads/min dose rate. Eight days after transplantation the animals were killed, the spleens fixed in Bouin's solution, and the spleen colonies counted indepently by two observers.

*Results. Intact mice.* No differences were found between the bone marrow cell counts of the testosterone-treated mice and the sesame oil control-treated mice. Both control and testosterone-treated animals had total cell counts per tibia ranging between 11.3 and 13.1 $\times$ $10^6$ on the first to seventh day of the study.

One day after testosterone treatment a moderate decrease in myeloid cells and a significant increase in erythroid cells were noted. The absolute numbers of myeloid cells were reduced from 4.8 $\times$ $10^6$ to 4.0 $\times$ $10^6$ per tibia ($P < 0.01$); erythroblasts rose from 3.2 $\times$ $10^6$ to 4.0 $\times$ $10^6$ cells per tibia ($P < 0.001$). Three days after treatment the total erythroid precursors were still above the control values, 3.7 $\times$ $10^6$ as compared to 2.6 $\times$ $10^6$ in control animals ($P < 0.01$). By the seventh day, erythroid cells had returned to control values.

The changes in the CFU-S levels of the two groups are shown in Fig. 1. The tibial marrow CFU-S content increased to 166% of control values ($P < 0.05$) on Day 3 after treatment and returned to normal range by the seventh day.

*Diffusion chamber studies.* Figure 2 shows the total number of nucleated cells harvested from the DCs. In the chambers of testosterone-treated hosts, the growth pattern of bone marrow cells showed an increase in total cell number as compared to the controls. After 3 days of culture the total cell count increased from 3.7 $\times$ $10^5$ in controls to 6.1 $\times$ $10^5$ in testosterone-treated animals ($P < 0.05$). Differential counts of

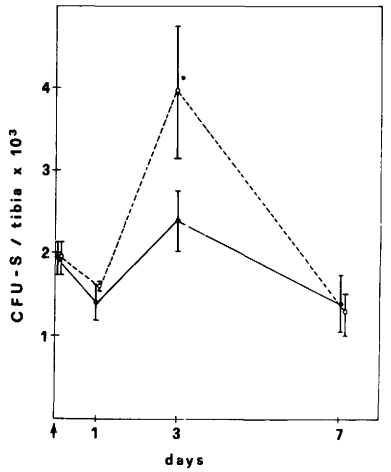


FIG. 1. The total number of CFU-S per tibia of Tstr-treated $CF_1$ mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). Values are means ± 1 SE of 7–14 samples from four separate experiments. (*) $P < 0.01$.

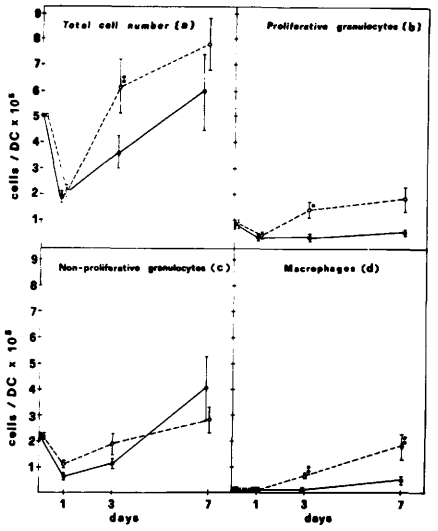


FIG. 2. (a) The total number of nucleated cells harvested from the DCs in Tstr-treated $CF_1$ host mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). (b)-(d) Changes with culture time of cell populations harvested from DCs. Values are means ± 1 SE of 6–11 samples from three to six separate experiments. (*) $P < 0.01$; (**) $P < 0.05$.

the cultured cells showed that erythroblasts declined soon after seeding; no difference was observed between control and steroid-treated animals. From the third day of culture onward, no nucleated erythroid cells were observed in the DCs. In DCs of testosterone-treated hosts, the number of proliferative granulocytes on Day 3 was significantly higher ($P < 0.05$) than controls, $1.4 \times 10^5$ and $0.46 \times 10^5$, respectively. A parallel increase in macrophages was observed on the third and seventh days of culture in the testosterone-treated animals. Lymphoid cells were present, but did not change in number throughout the culture period. Figures 2b, c, and d illustrate the growth curves of the major classes of cells present.

*Diffusion chamber CFU-S.* The CFU-S content of DCs implanted in testosterone and control-treated host mice is shown in Fig. 3. Although a marked decline in CFU-S was observed, levels of colony-forming cells in testosterone-treated hosts were significantly higher than in control animals on Days 1 and 3 of culture. No difference was observed after 7 days of culture.

*Discussion.* The numbers of cells harvested from 1-, 3-, and 7-day diffusion cultures showed an increased cellularity in testosterone-treated hosts. These changes were due to proliferation of both granulocytic cells and macrophages. In contrast, the tibial marrow cellularity did

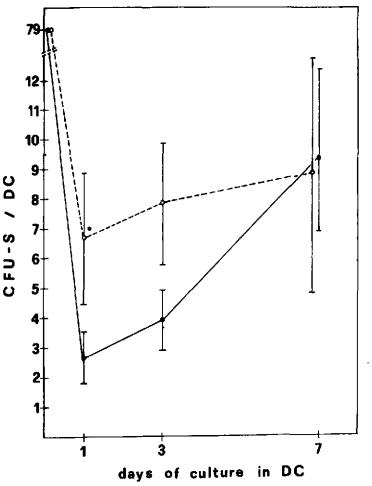


FIG. 3. The total number of CFU-S recovered in DCs implanted in Tstr-treated host mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). Values are means ± 1 SE of 10–23 DCs from four separate experiments. (*) $P < 0.1$.

not show any significant difference between the two treatment groups. This discrepancy observed between the DC and marrow cellularity can be explained by the fact that the DC system allows for proliferation and maturation of hemopoietic elements, but cellular migration is prevented (17).

In an *in vivo* environment, alterations in the marrow granulocyte reserve pool and granulocyte migration from the marrow could account for changes in total marrow cellularity. Although no increase in total cells was observed, detailed marrow differentials showed an increase in the percentage of erythroid elements 3 days after treatment. In contrast, the DC studies showed a sequential increase in dividing granulocytic cells and macrophages throughout the culture.

The results of *in vivo* CFU-S studies show that testosterone causes an increase in the number of CFU-S in the tibial marrow 3 days after injection. In the DC studies, injection of testosterone increases the number of recoverable CFU-S after 1 and 3 days of culture. Since both groups of animals showed a marked decline in chamber CFU-S, it is uncertain whether the observed increase in the testosterone-injected mice was caused by an enhanced proliferation rate or decreased cell loss at the level of the undifferentiated compartment. However, these data are in close agreement with those of Byron and Testa (18), Byron (15), and Fisher *et al.* (12), who showed that testosterone has a rapid *in vitro* effect which triggers CFU-S into cycle. The slower CFU-S growth phase found in the testosterone-treated group after the third day of DC culture (Fig. 3) may be related to CFU-S differentiation into granulocytic cells.

Studies have shown that testosterone causes an increase in plasma erythropoietin activity (1, 7, 8). Although ESF might affect the CFU-S by causing erythroid differentiation, several observations suggest that testosterone has a direct effect on hemopoietic stem cells. Testosterone causes increased proliferation of CFU-S in polycythemic animals in which there is no detectable ESF. Moreover, the stimulating effect of testosterone on marrow erythroid progenitor cells is not blocked by the simultaneous administration of anti-ESF serum (19).

The present data are consistent with the view that testosterone may have a direct effect on certain hemopoietic stem cells. In the DC system there is no erythroid differentiation, therefore the effect of testosterone on the CFU-S would not appear to be mediated by ESF. Instead, the enhanced numbers of CFU-S and granulocytic cells would appear to be due to a direct effect on the pluripotential and granulocytic stem cell compartments.

Rosenblum and Carbone (13) recently found increased numbers of colonies following *in vitro* addition of testosterone to cultures of human marrow. They attributed this effect to a direct stimulation of CFU-C proliferation. Our findings would be compatible with such a mode of action and, if this were the case, the observed granulopoietic effect of testosterone may have been due to its action on either the CFU-C or the CFU-S compartment.

In conclusion, we observed an absolute increase in CFU-S both in bone marrow and in diffusion chambers 3 days after injection of testosterone. The enhanced proliferation of the uncommitted progenitor cells is followed by increased bone marrow erythropoiesis and by granulocytic macrophage proliferation in the DC system. It is unclear whether testosterone acts at the CFU-S or at the committed compartment level, and further work is necessary to delineate the effect of this steroid on these stem populations. Our experimental data support the hypothesis that testosterone has more than one action on the hemopoietic system: by enhancing ESF production and by stimulating the stem cell populations. The further differentiation toward the granulocytic or erythroid lineage may be due to perturbations of the homeostatic mechanisms under various types of stress or injury and to the inductive action of the microenvironment as well.

*Summary.* Injection of long-acting testosterone markedly enhanced bone marrow CFU-S proliferation *in vivo* and CFU-

S recovery in a diffusion chamber (DC) system.

In the DC system the enhanced CFU-S proliferation led to increased cellularity due to an increase in dividing granulocytic elements and macrophages. *In vivo*, no difference was observed in the tibial total nucleated cellularity between treated and control animals. Detailed marrow differential counts, however, showed a significant increase in erythroid cell precursors 1–3 days after testosterone injection.

The relationship between colony-forming units and colony-forming cells is discussed and it is suggested that testosterone may act on different levels of the hemopoietic system. This steroid appears not only to enhance but also to induce proliferation of the uncommitted progenitor cells.

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