

# Androgenic Hormones and Human Granulopoiesis In Vitro

By Alan L. Rosenblum and Paul P. Carbone

**Androgenic steroids in physiologic concentrations were found to stimulate human granulopoiesis in vitro. Testosterone in high concentration, but probably within the range achievable after intensive treatment, was found to inhibit granulopoiesis.**

**Analysis of growth kinetics suggested that increased granulopoiesis results from proliferation of committed granulocyte precursors in the marrow rather than stem-cell activation.**

**A**NDROGENIC STEROIDS ARE thought to stimulate erythropoiesis both by increasing production of erythropoietin<sup>1</sup> and by direct effects on hematopoietic cells.<sup>2,3</sup> Clinical experience with androgens in the treatment of aplastic anemia<sup>4,5</sup> and during treatment with myelosuppressive agents<sup>6,7,8</sup> has suggested that these hormones may also stimulate granulopoiesis. In vitro<sup>9,10</sup> and in vivo<sup>10</sup> studies of granulopoiesis in testosterone-treated animals have indicated that leukopoiesis is increased, but no experimental model using human marrow cells has demonstrated that androgens effect granulopoiesis in humans.

Using the in vitro assay for granulocyte colony formation (CFU-C), we have investigated the response of normal human marrow with and without the addition of testosterone to the culture medium. An increase in CFU-C of 24%–60% was observed when the concentration of testosterone reached  $10^{-8}$ – $10^{-10}$  M, whereas inhibition of colony formation of 30%–35% occurred at concentrations of  $10^{-6}$  M or greater. These experiments confirm a stimulatory effect by androgens on human granulopoiesis. The inhibitory effects noted at higher concentrations may have implications for the clinical use of these compounds in hematologic disorders.

## MATERIALS AND METHODS

### *Collection of Human Marrow*

Normal bone marrow was obtained from volunteer subjects as part of the Normal Volunteer Program, Clinical Center, NIH. Four cubic centimeters of bone marrow was aspirated from the posterior iliac crest and transferred immediately to a plastic heparinized tube. A nucleated cell count and differential cell count were performed on the original sample. The marrow was spun in a narrow-caliber ("Dutch centrifuge") tube at 2700 rpm for 10 min and the resultant buffy coat removed. The marrow was washed twice in McCoy's + medium, spinning at 1500 rpm for 5 min after each wash. A nucleated count was then performed and the cell concentration adjusted for plating.

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### Plating and Counting

Cells in appropriate concentration were mixed with methylcellulose tissue culture medium containing a colony-stimulating factor (HEKS, Flow Laboratories), fetal calf serum, bovine serum albumin, and McCoy's + and then plated in 1.1-ml aliquots into each of four 35 × 10 mm plastic petri dishes as previously described.<sup>11</sup> Cultures were incubated for 10 days in a humidified atmosphere containing 7.5% CO<sub>2</sub> at 37° C. Plates were counted under an inverted microscope at 50 ×; any group of 50 or more cells was defined as one colony. A standard curve for each normal marrow was obtained by plating marrow at cell concentrations of 1.0, 1.5, and 2.0 × 10<sup>5</sup> cells per plate. When a single cell concentration was used to observe the effects of adding androgen, this concentration was usually 1.5 × 10<sup>5</sup> cells per plate. The effects of adding androgens were studied in both stimulated and unstimulated cultures. However, in our system, the unstimulated cultures produce few colonies (0-5), making statistical evaluation difficult. In this paper only stimulated cultures were used.

### Addition of Androgen

Prior attempts to observe increased colony formation in vitro by addition of testosterone to culture medium have used ethanol as a diluent, and we found in early experiments that alcohol was severely toxic to granulocyte colony formation. CFU-C was decreased by 70%-80% when as little as 100 μl of ethanol was added to 5.5 cc of culture medium. Testosterone is known to be soluble in propylene glycol, and we found that 5-50 μl of this diluent could be added to 5.5 cc culture medium without observable effects on colony counts.

Crystalline testosterone propionate was dissolved in propylene glycol diluent in varying concentrations and added in 5-μl volumes to each culture tube of 5.5 cc prior to plating. All concentrations noted refer to the final concentration of testosterone after mixing with culture medium. Controls containing propylene glycol alone and without drug or diluent were included with each experiment.

### Calculation and Statistical Analysis

For each experiment the number of colonies on each of four replicate plates was averaged and a mean and standard error determined. Evaluation of effects due to addition of drug or diluent was made by relating the colony count to the experimental group to the standard curve for the control to derive an "effective" cell concentration. By comparing the effective cell concentration of the experimental group to the cell concentration of the control, a per cent stimulation or inhibition was calculated as follows:

$$1 - \frac{\text{Effective experimental concentration}}{\text{Cell control concentration}} \times 100$$

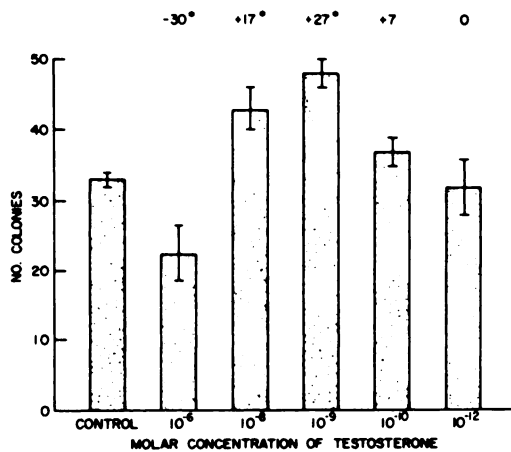


Fig. 1. Representative dose-response curve for testosterone using marrow from normal female. Bars indicate colony count ± standard error. Figures above bars indicate degree of inhibition (-) or stimulation (+). Asterisk (\*) indicates those levels which differ from control at  $p > 0.05$  level. (Note that concentrations of testosterone are plotted from high to low doses.)

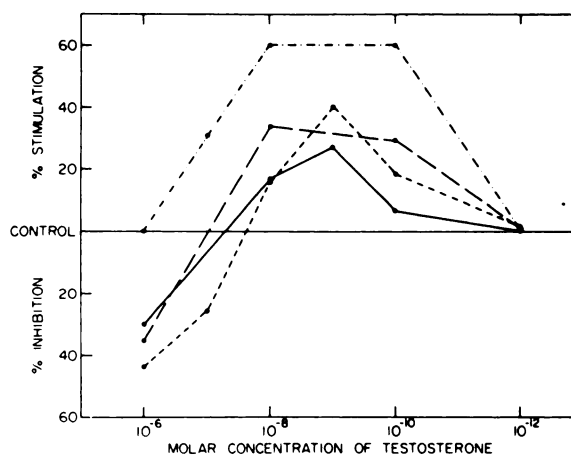


Fig. 2. Dose-response curve for testosterone from four different marrows.

## RESULTS

The effects of adding testosterone in concentrations of  $10^{-12}$ - $10^{-6}$  M on granulocyte colony formation of a culture from normal female stimulated with HEKS and testosterone are shown in Fig. 1 for  $1.5 \times 10^5$  cells plated. At concentrations of  $10^{-6}$  M there was a significant inhibition ( $-30\%$ ) ( $p < 0.05$ ). At concentrations of  $10^{-8}$  and  $10^{-9}$  M,  $17\%$ ,  $27\%$  stimulation was observed ( $p < 0.05$ ). In this experiment, at  $10^{-10}$  and  $10^{-12}$  M no effect, as compared to a control culture without testosterone, was seen.

The results of four separate marrows cultured with similar concentrations of testosterone are shown in Fig. 2. Consistent patterns of stimulation and inhibition were seen. At concentrations of  $10^{-6}$  three of four marrows had definite evidence of inhibition ( $30\%$ ,  $45\%$ ). At concentrations of testosterone of both  $10^{-8}$  and  $10^{-12}$  there arose  $10\%$ - $60\%$  stimulation, as measured by increased numbers of colonies. At  $10^{-12}$  M the colony numbers were back to control levels.

The effects of adding a single concentration of testosterone to varying concentrations of marrow cells can be seen in Fig. 3. In these experiments the

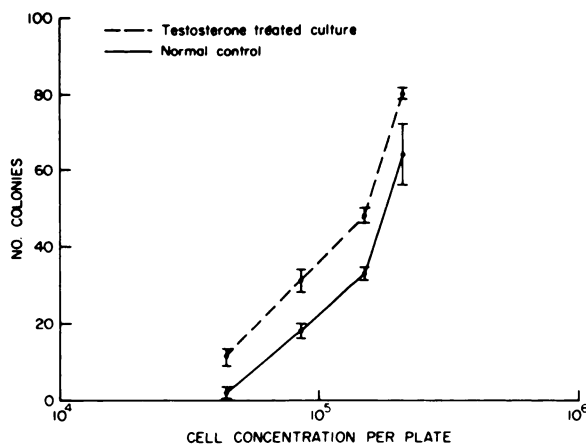


Fig. 3. Effect of single-dose level of testosterone ( $10^{-9}$  M) on cell dose-response curve of normal marrow. Note similar increase in colony count (approximately 14 colonies) at each cell-dose level as a result of adding testosterone. (Note that concentrations of testosterone are plotted from high to low doses.)

standard curve appears to be shifted to the left as a result of a similar increase in the number of colonies at each dose level.

There were no significant differences between the response of marrow obtained from male versus female subjects. There were no observable differences in colony morphology due to addition of testosterone; however, colony size appeared greater in those cultures showing marked stimulation.

#### DISCUSSION

The increase in colony-forming ability observed with testosterone could result either from increased numbers of granulocyte progenitor cells induced to form colonies *in vitro* or from differentiation of a more primitive cell into the committed granulocyte progenitor cell pathway. It has been established that colonies arise *in vitro* from committed granulocyte precursors, and it would be important to establish whether increased colony number was the result of stem cell activation or stimulation of committed granulocyte precursors. We attempted to evaluate these alternatives by observing the effects of androgens on the cell dose-response characteristics of marrow. If increased colony formation was the result of stem cell recruitment, an increasing number of colonies would be expected as a consequence of increasing the concentration of cells plated. For example, if 0.01% of the marrow consists of "recruitable" stem cells, then at concentrations of  $5 \times 10^4$  an additional five colonies would be observed, and at concentrations of  $5 \times 10^5$  an additional 50 colonies should appear. In fact, we noted a fixed increase in colony number regardless of cell dose plated (i.e., the standard curve was shifted to the left), which we interpret as resulting from proliferation of already committed granulocyte precursors. This increase in absolute colony count was related to the dose of androgen plated and appeared maximal in the range of  $10^{-9}$  M.

It is possible that stem cells are also affected by testosterone, but in the *in vitro* assay, this activation did not result in increased colony formation. In mice, testosterone produces an absolute increase in CFU-S in mouse femoral marrow 24 hr after treatment,<sup>12</sup> and after 6 hr there is an increase in the sensitivity of CFU-S to the cytotoxic action of tritiated thymidine.<sup>13</sup> This rapid action of testosterone may result from a shortened cell cycle time or a stimulation of resting stem cells into cell cycle. Whether such activation of CFU-S leads to increased granulopoiesis *in vivo* was not studied.<sup>13</sup>

The mechanism whereby androgens are capable of stimulating granulocyte precursors remain unclear. Androgenic hormones have been shown to stimulate leukopoiesis in irradiated rats by increasing marrow production.<sup>14</sup> Testosterone administration for 3 days to mice increases the number of CFU-C that can be obtained from marrow by 50%–90%, and this increase in CFU-S is associated with a simultaneous 60%–70% increase in DNA synthesis by marrow cells.<sup>10</sup> Mouse marrow cells incubated with testosterone show an increased susceptibility to subsequent inactivation of ARA-C, an inhibitor of DNA synthesis.<sup>10</sup> These findings suggest that testosterone increases the proportion of granulocyte precursor cells entering DNA synthesis by altering the growth fraction. Our data is most consistent with the hypothesis that committed granulocyte progenitors are recruited from a pool already present in the plated marrow

but which, under conditions present in culture, do not proliferate. Testosterone is capable of inducing proliferative activity in this population by affecting events early in cell cycle and resulting in increased colony-forming ability.

Testosterone may directly stimulate granulocyte progenitor-cell proliferation or may serve a permissive role by affecting sensitivity to colony-stimulating factor (CSF) or affecting the adherent or colony-stimulating cell. Techniques for studying the cells have been recently reported.<sup>15</sup> A similar permissive mechanism has been proposed for the action of androgens in erythropoiesis where stem cell sensitivity to erythropoietin is increased by androgen and erythroid differentiation is enhanced.<sup>16</sup>

The inhibitory effects of high testosterone concentrations should be noted. The normal level of testosterone in human plasma by chromatographic or double-isotope dilution methods approximates 26–108  $\mu\text{g}/100\text{ ml}$  for females and 273–1211  $\mu\text{g}/100\text{ ml}$  for males.<sup>17</sup> This corresponds to a plasma concentration from 0.1 to  $3.0 \times 10^{-8}\text{ M}$ . From data related by Aavag and Vogt,<sup>18</sup> a single intramuscular dose of testosterone enanthate of 250 mg will result in plasma levels as high as 4.0  $\mu\text{g}/100\text{ ml}$  or  $10^{-7}\text{ M}$ . It would thus appear possible to achieve plasma levels of  $10^{-6}\text{ M}$  in humans following intensive testosterone treatment. If these levels are indeed inhibitory to either granulocyte progenitor cell proliferation or to stem cells per se, in vivo as they appear to be in vitro, then careful monitoring of testosterone levels should be undertaken in these patients. The effects of prolonged high-dose androgen therapy on granulopoiesis require further investigation.

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