The effects of testosterone and erythropoietin (ESF) on erythroid colony formation in normal human bone marrow cultures were studied in vitro using a methyl cellulose gel system. Testosterone was found to produce a significant increase in erythroid colony formation at concentrations of $10^{-4}$-10$^{-8}$ M in vitro. In this system, the numbers of erythroid colonies formed per plate increased in direct proportion to the increase in the number of erythroid precursors inoculated as well as to the increase in the dose of ESF in vitro.

In addition, a synergistic effect of a combination of testosterone and ESF on erythroid colony formation was seen when ESF was present at high concentrations. These data suggest that a greater number of erythropoietin-responsive cells are available for ESF to differentiate into the nucleated erythroid cell line in the presence of testosterone, indicating that the effect of a combination of testosterone and ESF is greater in enhancing erythropoiesis than the additive effects of either agent alone.

It has been demonstrated by several investigators that androgens stimulate erythropoiesis in animals and in man. It seems clear that the erythropoietic effects of androgens are, at least in part, due to their ability to increase erythropoietin (ESF) production. Recently, some investigators have suggested that androgens not only stimulate ESF production but also act directly on hematopoietic stem cells (CFU). We have demonstrated previously, using a methyl cellulose gel system, that testosterone produces a significant increase in erythroid colony formation in rabbit bone marrow cultures in vitro. However, possible direct effects of androgens on human bone marrow erythroid cells are not well understood. In addition, the role of ESF in androgen-induced erythropoiesis is not clear and has not heretofore been investigated. It seems important, in considering therapy for patients with refractory anemias, such as the anemia of renal insufficiency, to determine whether the administration of ESF and/or androgens increases erythroid colony formation in human bone marrow cultures.

In the present studies, the response of normal human marrow cells to either ESF or testosterone alone or to a combination of both agents was investigated in vitro using a modification of the method of Iscove et al. for studying erythroid colony formation in a methyl cellulose gel system. The number of erythroid colonies formed is considered to be directly proportional to the number of erythropoietin-responsive cells (ERC) in the bone marrow sample in-
oculated. Testosterone was found to produce a significant increase in the numbers of erythroid colonies in human bone marrow cultures at concentrations of $10^{-4}$ to $10^{-3} \, M$ in vitro. In addition, the combination of ESF and testosterone caused an increase in the numbers of erythroid colonies which was more than additive.

**MATERIALS AND METHODS**

Bone marrow was obtained from normal human volunteers with their proper consent. Approximately 2 ml of bone marrow was aspirated from the posterior iliac crest and transferred immediately to a sterile plastic heparinized tube containing 10 ml alpha-medium (Flow Laboratories, Inc., Inglewood, Calif.). Nucleated cell counts and differential cell counts were performed on the bone marrow sample. The marrow was centrifuged at 1500 rpm for 15 min, the fatty tissue removed, and the buffy coat collected. In order to remove as many granulocytes from the marrow cells as possible, the buffy-coat cells (total of $5 \times 10^7$ red plus nucleated cells) in 10 ml alpha-medium containing 25% fetal calf serum (Grand Island Biological Company, Grand Island, N.Y.) was placed in a plastic 100-mm tissue culture dish, and incubated at 37°C in 5% CO$_2$ for 1 hr. The suspended nonadherent cells were then pipetted into a plastic tube. The marrow was washed once in alpha-medium, a nucleated cell count was performed again, and the cell concentration adjusted for plating.

Cultures of erythroid colonies were carried out according to a modification of the method of Iscove et al. as follows: 2 x $10^5$ nucleated marrow cells, from which granulocytes had been removed as described above, were plated on 35 x 10-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.) in 1 ml alpha-medium containing 0.8% methyl cellulose, 30% fetal calf serum, 1% deionized bovine serum albumin, penicillin (50 U), and streptomycin (20 μg). No detectable erythropoietic activity was seen when 1.0 ml of this culture medium was assayed in exhypoxic polycythemic mice.

Testosterone (testosterone hemisuccinate sodium salt, kindly supplied by Dr. John Babcock of the Upjohn Co., Kalamazoo, Mich., was dissolved in alpha-medium containing 10% fetal calf serum in varying concentrations and added in 5-μl volumes to each culture plate prior to plating, with and without the addition of human urinary erythropoietin* (0.02-0.2 U per plate), with a specific activity of 5.29 U/mg, in a 5-μl volume of alpha-medium. Both testosterone and erythropoietin were sterilized by passage through Millipore filters (0.45 μ) before addition to the culture plates. Three plates were prepared for each group.

The bone marrow cultures were incubated for 4 days at 37°C in an incubator with a humidified atmosphere of 5% CO$_2$ and 95% air. Erythroid colonies containing ten or more cells were scored on one-fourth of the total plate area using an inverted microscope at 75 x magnification. Erythroid colonies were identified after staining with benzidine.

For each experiment the number of erythroid colonies on each of three replicate plates were averaged and the mean and standard error determined.

**RESULTS**

The effects of testosterone in concentrations of $10^{-3}$ to $10^{-8} \, M$ on erythroid colony formation in bone marrow cultures from normal human subjects, using 2 x $10^5$ cells are shown in Table 1. At a concentration of $10^{-3} \, M$, there was a slight, but not a statistically significant, inhibition ($-9\%$) of colony formation as compared with controls. At concentrations of $10^{-4}$ to $10^{-8} \, M$, an increase in erythroid colonies was seen which ranged between 22% and 85%. The most

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*The erythropoietin was collected and concentrated by the Department of Physiology, University of The Northeast, Corrientes, Argentina, and further processed and assayed by Hematology Research Laboratories, Childrens Hospital of Los Angeles, Los Angeles, Calif., under Research Grant HE 10880 (National Heart and Lung Institute).
Table 1. Effects of Testosterone on the Formation of Erythroid Colonies in Normal Human Bone Marrow Cultures

<table>
<thead>
<tr>
<th>Molar Concentration of Testosterone</th>
<th>No. of Experiments*</th>
<th>No. of Colonies Per One-quarter Plate</th>
<th>Per Cent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (8)</td>
<td>19.6 ± 2.6†</td>
<td>-9</td>
<td></td>
</tr>
<tr>
<td>10⁻³</td>
<td>(4)</td>
<td>17.8 ± 2.9</td>
<td>-9</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>(4)</td>
<td>24.0 ± 4.6</td>
<td>+22</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>(4)</td>
<td>32.9 ± 5.8</td>
<td>+68†</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>(4)</td>
<td>36.3 ± 4.2</td>
<td>+85†</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>(4)</td>
<td>34.7 ± 3.6</td>
<td>+77†</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>(4)</td>
<td>33.4 ± 4.9</td>
<td>+70†</td>
</tr>
</tbody>
</table>

*Each experiment (4–8) represents the mean number of colonies for three replicate culture plates.
†Standard error of mean.
‡Significantly (p < 0.01) different from control.

marked and statistically significant (p < 0.01) increase was seen at concentrations of 10⁻⁶ and 10⁻⁷ M.

The results of the addition of a single concentration of ESF (0.02 U) to varying concentrations (1–8 × 10⁴ cells) of marrow cells in cultures are shown in Fig. 1. In these experiments, the increase in erythroid colonies formed was directly proportional to the increase in erythroid precursors in the normal human marrow cultures between 1 and 8 × 10⁴ cells.

In order to determine the role of ESF in androgen-induced erythropoiesis, changes in the number of erythroid colonies in cultures with varying concentrations of ESF, using 2 × 10⁵ cells, were studied with and without testosterone (10⁻⁶ M). As illustrated in Fig. 2, the numbers of erythroid colonies in human bone marrow cultures increased in proportion to the increase in the concentration of ESF, reaching a plateau at 0.2 U of ESF. It is interesting to note that an increase in the numbers of erythroid colonies in cultures was seen, when ESF was present at high concentrations, with the combination of testosterone and ESF which was more than additive, as noted from the derived curve which was the sum of the effects of testosterone alone at 10⁻⁶ M and a given

Fig. 1. Regression line for the number of erythroid colonies formed per plate versus the number of marrow cells inoculated. Each culture plate contained 0.02 U of human urinary erythropoietin. Each point indicates mean ± SEM (four experiments).
dosage of ESF. These data suggest a synergistic action between ESF and testosterone. There was not a detectable difference in the morphology of the erythroid colonies formed due to the addition of testosterone and the controls. However, the colony size appeared to be greater in the cultures incubated with ESF.

DISCUSSION

The results of the present studies indicate that testosterone stimulates erythroid colony formation in normal human bone marrow cultures, confirming our previous observations of an increase in the number of erythroid colonies in rabbit bone marrow cultures following in vitro and in vivo stimulation with testosterone. Erythroid colony-forming ability of human bone marrow, assessed with the use of a methyl cellulose gel system, indicated that the increase in erythroid colonies formed in the cultures is proportional to the increase in the number of precursors inoculated in the presence of ESF. The ability of ESF to stimulate erythroid colony formation using this culture method has been confirmed by other investigators. This culture system may be useful as a technique for evaluating the function of erythroid stem cells, as well as the number of erythropoietin-responsive cells (ERC), by determining the number of erythroid colonies formed in the cultures.

There was significant basal spontaneous growth of erythroid colonies in our human bone marrow cultures with the methyl cellulose system without the addition of erythropoietin. However, we cannot be sure that an amount of ESF is present in the culture media which is not detectable in the biologic assay. It is of interest that spontaneous growth of erythroid colonies was also seen, with the plasma clot method without the addition of erythropoietin to the culture medium, in the 4-day fetal mouse liver. It is possible that some ERC have already been triggered by ESF in vivo to cycle before their removal from the rabbit and continue to replicate in the in vitro culture system.
Several reports have appeared during the past few years which have attempted to clarify the mechanism by which androgens stimulate erythropoiesis in animals and in human subjects. It seems clear that androgens increase erythropoietin production in man and experimental animals. In addition, the antibody to ESF was found to block the erythropoietic effects of testosterone. However, a possible direct effect of androgens on human bone marrow erythroid cells is not as well understood. In addition, a clarification of the role of ESF in androgen-induced erythropoiesis may provide a more rational basis for the treatment of patients with refractory anemia, such as anemia of renal failure. Certain 5β-H steroid metabolites have been reported to increase heme and globin synthesis in human bone marrow cultures, but an increase in heme synthesis was not seen with testosterone. Similarly, Necheles has suggested that etiocholanolone may act as an inducer of heme synthesis in human bone marrow cultures and thereby regulate the differentiation of hematopoietic stem cells (CFU) into erythroid precursors. However, heretofore there have been no reports of a direct effect of androgens on erythroid colony formation in human bone marrow. The mechanism whereby testosterone stimulated erythroid colony formation is not clear. Our finding in the present studies of a significant increase in the numbers of erythroid colonies in normal human bone marrow cultures stimulated by testosterone may be explained by a direct action of testosterone on the hematopoietic stem cell compartment (CFU). This effect on the uncommitted stem cells may cause their differentiation into the ERC compartment. This finding is similar to that obtained in the in vitro assay (previously reported) for erythroid colony formation in rabbit bone marrow cultures where the addition of the alkylating agent, busulfan, to the culture medium blocked the effects of testosterone on colony formation but did not block the effects of ESF in enhancing formation of erythroid colonies. Furthermore, the increase in the number of erythroid colony-forming cells seen following stimulation by testosterone in vivo was completely inhibited by the prior administration of busulfan. It has been postulated from these studies that busulfan blocks the formation of new ERC from the CFU pool. In addition, in the present studies, a synergistic effect of a combination of testosterone and ESF on erythroid colonies was seen, especially at higher dosages of ESF (Fig. 2). This suggests that more ERC are available for ESF to differentiate into erythroid cells, indicating that a combination of testosterone and ESF is more effective than either agent alone in enhancing erythropoiesis.

It is possible that stem cells are also affected by testosterone in the in vivo assay. Testosterone produces an absolute increase in CFU-S in the femoral bone marrows of mice 24 hr after testosterone treatment, and after 6 hr there is an increase in the sensitivity of CFU-S to the cytocidal action of tritiated thymidine. This action of testosterone may result from a shortened cell cycle time or a stimulation into cell cycle of resting stem cells. Even though we postulate that testosterone acts on CFU to cause their differentiation into the ERC compartment, consequently enhancing erythropoiesis, it is still possible that, in addition to this effect of testosterone on uncommitted stem cells, it could also act upon ERC. However, we feel that the synergistic effect of the combination of testosterone and ESF demonstrated in the present studies suggests that
the primary effect of testosterone is to induce differentiation of CFU to increase the population of ERC in the presence of high concentrations of ESF in cultures. The increase in colony size seen with high doses of ESF may be due to an increase in the differentiation of ERC, causing an increase in the numbers of nucleated erythroid cells in cultures stimulated by ESF.

REFERENCES

5. Moriyama Y, Fisher JW: Increase in erythroid colony formation in rabbits following the administration of testosterone. Science (submitted)
Effects of testosterone and erythropoietin on erythroid colony formation in human bone marrow cultures

Y Moriyama and JW Fisher