Tissue Culture of Bone Marrow
II. Effect of Steroid Hormones on Hematopoiesis in Vitro

By Edward H. Reisner, Jr.

The nature of the mechanisms regulating hematopoiesis is obscure. There is much evidence that humoral factors are involved and that they are of a hormonal nature. In particular, the steroid hormones of the gonads and adrenal cortex have been shown to have effects on the growth of cells in vitro\(^1,2\) and on hematopoiesis in vivo\(^3,4\). The clinical usefulness of testosterone in the treatment of patients with aplastic and aregenerative anemias\(^5\) and of cortisone in patients with leukemia\(^6\) is well established.

Development of a tissue culture technique which could maintain marrow for appreciable periods of weeks to months producing recognizable blood cells without fibroblastic overgrowth\(^7\) facilitated the study of the effect of specific substances on blood cell formation. This paper reports the result of such studies with prednisolone, testosterone and estrone. Prednisolone was chosen because it was known to inhibit fibroblast formation in vitro as well as through its clinical effect on leukemia, testosterone because of its apparent erythrostimulatory effect, and estrone as a natural antagonist to testosterone to serve as a control.

Methods

Normal marrow was obtained from ribs freshly resected from patients undergoing thoracic surgery. Explants of approximately 5 mm. diameter were placed in the bottom of a well formed by sealing one end of a glass cylinder 15 mm. in diameter and 10 mm. high with a 22 mm. square glass cover slip. A tantalum gauze mesh, cut to fit the inside area of the well, was placed over the explant, medium was added to a depth of 8 mm., and the well sealed with a second cover slip. Medium consisted of balanced salt solution (NTCA 109 or mixture 199) and 20 per cent horse serum. To this was added 0.001-0.005 mg./ml. of an aqueous suspension of testosterone, estrone and prednisolone phosphate. Wells were sacrificed every few days for a period of observation of 3 weeks. Romanowsky-stained preparations were made of the air-dried bottom cover slip and explant. If the latter was large, preliminary fixation with methyl alcohol was sometimes done. In some experiments 0.1 μc./ml. of H\(^3\) thymidine was added to the culture for 24 hours and radioautographs were prepared from the bottom cover slips.

In these cultures the different cell types exhibit characteristic growth patterns. Fibroblasts develop from free floating mononuclear cells, reticulum cells and immature granulocyte precursors around the edges of the explants. They appear first and in the greatest abundance in the more sparsely cellular areas. They are metabolically active cells as shown by the rapid acidification of the medium in cultures in which they are numerous, and their rich content of enzymes of the glycolytic pathways. They contain considerable amounts of alkaline phosphatase. Under conditions which favor fibroblastic growth, round,
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undifferentiated cells with pale, often vacuolated cytoplasm are also frequently present. They have a superficial resemblance to macrophages but do not exhibit phagocytic properties to any significant degree. They often grow in clumps or sheets and for this reason we have called them epithelioid cells. Occasionally, multinucleated giant cells are seen which appear to arise from the fusion of several epithelioid cells. When fibroblasts are removed from a glass surface with trypsin and resuspended, they become round and resemble epithelioid cells, and for some years we regarded the latter as a different morphologic form of fibroblast. However, the epithelioid cells do not contain demonstrable alkaline phosphatase and would therefore appear to differ metabolically as well as structurally from the spindle-shaped fibroblasts.

Granulocytes grow in characteristic clumps varying in size from a few cells to hundreds (fig. 1A). In vigorously growing cultures, such clumps are regularly seen and we consider their presence an index of active granulopoiesis. The clumps consist of one or more large mononuclear cells around which are recognizable granulocyte precursors. Mitotic figures may be seen among these peripheral cells, which label with tritiated thymidine in contrast to the large cells in the center. Time-lapse cinematography by the late Dr. Charles Pomerat confirmed the presence of vigorous mitotic activity at the periphery of similar clumps of leukocytes induced by phytohemagglutinin. Initially the cells in the clumps contain characteristic cytoplasmatic granules, but after a week or so in culture the cytoplasm becomes paler and often vacuolated. Granulopoietic clumps occur within the explant of marrow or around its edges.

In contrast, foci of erythropoiesis tend to be confined to main explant or to small pieces of it that become separated. The obvious explanation for this difference is the lack of motility of erythroid cells. Erythropoietic foci may be found in the wall of proliferating marrow sinusoids (fig. 2B) where these are preserved in the cultures. They are easily recognizable by the homogeneity of the compact nuclei and the presence of hemoglobin. Although mitotic figures are rarely seen in erythropoietic foci, the earlier forms can be labeled in vitro without difficulty.

Megakaryocytes may be seen floating free or developing from sinusoidal endothelium. They label well with H3-thymidine and show polyploid nuclei as would be expected. In vigorously growing marrow cultures the marrow fat is rapidly depleted and the stromal elements of the explant are obliterated by the proliferating cells. The disappearance of the stroma in a vigorously growing culture is so rapid (2 or 3 days) that it suggests some type of lytic action.

RESULTS

The results, summarized in table 1, indicated a stimulation of granulopoiesis by estrogens, erythropoiesis by androgens and inhibition of fibroblast, and epithelioid cell formation by prednisolone.

Because of the variations in intrinsic cellularity and growth potential between individual marrows, differences between cultures should not be considered significant unless they can be reproduced in marrow specimens from several different individuals, grown under the same conditions. In the present series of experiments qualitatively similar results were observed in all the cultures that grew satisfactorily. Quantitation of results in these cultures was not feasible, because the total number of cells of different types present in each explant at the beginning of the experiment could not be determined. If the medium surrounding the explant contained many cells, it was possible to perform differential counts reflecting changes going on in the explants. Results of such counts for four pairs of wells from two experiments are shown in table 2 and reflect the qualitative impression of enhancement of erythropoiesis by androgens and granulopoiesis by estrogens.
Controls

In this series of experiments the controls showed a significant degree of fibroblast and epithelioid cell formation after 2–3 days. This appeared in the less cellular areas peripheral to the explant and was well established by the end of a week. It did not overgrow the culture and granulocytic, erythro-
Table 1.—Qualitative Evaluation of Hormonal Effects on Marrow in Vitro

<table>
<thead>
<tr>
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<th>Proliferation of Fibroblasts, Leukocytes and Erythroid</th>
<th>Preservation of Stroma &amp; Blood Vessels</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td>Estrone</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Testosterone</td>
<td>-</td>
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</tr>
<tr>
<td>Prednisolone</td>
<td>-</td>
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</table>

Table 2

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Time</th>
<th>Estrogen</th>
<th>Androgen</th>
</tr>
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<tr>
<td>80</td>
<td>6 days</td>
<td>2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>80</td>
<td>10 days</td>
<td>5.7</td>
<td>1.5</td>
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<tr>
<td>85</td>
<td>4 days</td>
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<td>0.7</td>
</tr>
<tr>
<td>85</td>
<td>8 days</td>
<td>5.6</td>
<td>1.7</td>
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Ratio of granulocytic cells/erythroid cells in medium adjacent to marrow explants cultured in medium containing estrone or testosterone (10 fields of 100 cells counted in each culture).

cytic and reticulum cells, and megakaryocytes continued to form in the explants and be released into the surrounding medium for the duration of the experiment. The amount of fibroblast formation varied with the individual marrows used in each experiment, as did the original proportions of myelocytic to erythroid elements. In some cultures granulopoiesis was active and rapidly destroyed the original marrow architecture (fig. 3A).

Experimental

*Estrone.* In 22 of 25 experiments with estrone there was marked or moderate granulocytic activity (figs. 1A, 2A). Granulocytes grow in clusters around one or several reticulum cells, a phenomenon which was observed in most of the cultures (fig. 2A) (one culture failed to grow and two became contaminated.) Fibroblast formation was less prominent than in the controls and absent in some cultures. All estrone cultures were highly cellular. In the radioautographs, labeling of all elements was seen. In the clumps the central reticulum cells did not label, but good labeling was observed in the cells around them.

*Testosterone.* In 20 of 25 experiments (5 were discarded because of failure to grow, contamination or loss of one control), inhibition of fibroblast growth was complete or almost so. Explants were, in general, less cellular than controls with better preservation of the stromal and endothelial elements. Erythropoietic foci (fig. 1B, 2B.) were conspicuous in the explants and there were increased numbers of hemoglobinated cells in the surrounding medium. In some cultures erythropoiesis was so marked that the entire stained explant had a reddish hue when viewed under the low power of the microscope. That this was due to hemoglobin was proved by specific staining using the method of Gross. Labeling of all elements was observed.

*Prednisolone.* In more than 50 experiments with prednisolone (including some done coincidental to other studies), inhibition of fibroblast formation
was complete or nearly so. Explants preserved their architecture (fig. 3B) but became much less cellular. Endothelium in the sinusoids was nonproliferative. There was continued differentiation of all elements of the blood but fewer cells were produced. Many of the cells appeared to be dead or dying. Good labeling of early forms of all blood lines was observed with H3 thymidine.

Fig. 2A.—Culture 85 (A) 4 days—estrone; (B) 8 days—testosterone. Erythropoietic foci in walls of sinusoids. (160 ×)
Fig. 3A.—Culture 78—5 days. (A) Control showing vigorous growth and only a small fragment of explant remaining which is almost completely overgrown; (B) prednisolone showing preservation of original marrow explant architecture. (65 ×)

Fig. 3B.—See legend under Figure 3A.

DISCUSSION

The mechanisms regulating the proliferation and differentiation of blood cells are still imperfectly understood, despite the many advances in methodology achieved in recent years. Homeostatic regulation of blood levels requires a flexible humoral control system in at least three stages of blood for-
mation: (1) the stem cell division, (2) the division of differentiated and maturing cells, (3) the destruction of cells before they reach the circulation. Each of these steps is probably subject to humoral regulation for which some of the evidence may be summarized. With regard to (1), the classic example is the ability of adrenal corticosteroids to clear the marrow of blasts in patients with acute leukemia. For (2), the erythrostimulatory effects of erythropoietin, both in vivo and in vitro, and the influence of androgens on erythrocyte levels may be cited. For the third stage postulated, kinetic studies with radioactive tracers have made it abundantly clear that in many conditions there is more intramedullary cell formation taking place than can be subsequently accounted for in the peripheral circulation, the missing cells apparently being destroyed before they leave the marrow by enzymatic, immunologic or other cytolytic mechanisms.

The observations reported in this paper are consistent with these postulates and may be considered in their light. The inhibitory effect of cortisone and prednisolone on fibroblast formation in tissue cultures has been previously reported.2,10 (A recent paper by Fames and Barker11 reported stimulation by prednisolone of fibroblast growth from marrow aspirates growing in wells under conditions generally comparable to our own. The reason for this contradictory observation is not apparent, but it may be related to the fact that she used aspirates which contain many more free-floating cells which have a greater tendency to form fibroblasts.) Cohen and Gardner12 reported depressed erythropoietic response and preservation of marrow fat in rabbits receiving phenylhydrazine and triamcinolone. Their in vivo observations correlate well with our observations of the suppressive action of prednisolone in vitro. Nowell’s studies13 led him to believe that prednisolone did not inhibit mitosis of leukocytes in vitro, but rather interfered with the conversion of partially differentiated leukocytes to a state capable of mitosis. The growth of fibroblasts in marrow cultures is in some ways analogous to the growth of blasts in the marrow in leukemia.* It is possible that the property of cortisone that inhibits fibroblast formation and allows normal hematopoiesis to proceed at a slow rate in vitro is the same one that can restore normal hematopoiesis in the marrow of the leukemia patient. If one regards the lymphocyte as a potential stem cell, the known lymphocytolytic action of cortisone makes such a hypothesis all the more attractive. In the cultures with prednisolone the endothelial structures, from which the hemic stem cells develop, are much better preserved without evidence of significant proliferation of cells. It may be that as the result of virus infection, irradiation injury (or genetic predisposition) leukemic cells produce humoral substances that influence their own metabolism in the direction of continued division and away from differentiation. In this connection the observations of Dougherty14 on the abnormal metabolites of cortisol produced by leukemic tissue in vitro appear significant.

*Our observations have led us to consider the “fibroblast” to be an undifferentiated cell of endothelial or hemic origin structurally modified by growth upon a surface, with the ability to proliferate under favorable conditions.7
Testosterone also inhibited fibroblasts, but in addition it created a metabolic milieu more favorable for erythropoiesis. This would be explicable in the proposed schema by an inhibitory action at the stem cell level, and a stimulatory action at the second stage.

At the start of these experiments estrogen was chosen as a natural control for the testosterone experiments. The strong stimulus to granulopoiesis was unexpected. Review of the literature yielded several reports of similar activity of estrogenic hormones. Von Haam and Cappel reported that estrogens in higher dilutions stimulated and in greater concentrations did not inhibit fibroblast growth in vitro in contrast to androgens. Kelly found estrogens stimulated fibroblast growth in sponges implanted subcutaneously in rats. Bimes et al. noted that estradiol and testosterone inhibited HeLa cell growth in vitro but not chick fibroblasts or uterine endothelial cells. Fox noted faster differentiation of granulocytes in marrow cultures from mice pretreated with estrogen. In vivo estrogens have been shown to inhibit erythropoiesis in rats and hamsters and to increase the incidence of lymphoid tumors in C3H mice and induced (x-ray or mecholanthrene) leukemia in BALB, CDA and DBA mice. The incidence of x-ray-induced leukemia in BC mice was enhanced by estradiol and inhibited by testosterone. The action of estrone in our experiments would appear to be confined to a stimulatory effect on granulopoiesis at stage 2. It may be asked whether this is merely an apparent stimulation of granulopoiesis due to a suppressive action on erythropoiesis. There are several arguments against this suggestion. Erythropoiesis was not absent in the estrone cultures but was simply overshadowed by granulopoiesis. The leukocytes grew in characteristic clusters, similar to those produced in vitro by other stimulants to leukocyte proliferation such as phytohemagglutinin. Fibroblast growth was not inhibited but was overshadowed by the leukopoiesis.

While the effects in vitro of the hormones studied were easily demonstrable, we can only speculate on their mode of action. Fibroblasts have the greatest amount of respiratory activity as evidenced by the rate of acid formation in the cultures; leukocytes are also active in this respect. In contrast, the cultures with testosterone and prednisolone change pH only slowly. One may hypothesize that the hormonal action is to depress the activity of enzymes essential for cell respiration. That different types of blood cells thrive only in favorable environments related to the availability of oxygen and nutrients per cell is the basis of Osgood’s gradient principle theory of cell growth. A granulocyte with a higher respiratory activity would not grow in a metabolic environment that could support an erythroblast with a lower energy requirement. Studies are underway to investigate this point. The hypocellularity of the cultures with prednisolone also suggest that it may have a direct action on the formation of stem cells from the marrow endothelial structures.

**Summary**

In cultures of normal human bone marrow in a medium of 20 per cent horse serum and balanced salt solution, estrone stimulated granulopoiesis; testos-
terone inhibited fibroblast formation markedly and stimulated erythropoiesis; prednisolone inhibited fibroblast formation completely and allowed continued differentiation of all blood elements in smaller numbers.

**Summario in Interlingua**

In culturas de normal medulla ossee human in un medio de 20 pro cento de sero equin e de 80 pro cento de balanciate solution salin, le sequente observationes esseva facite: Estrona stimulava le granulopoiese; testosterona inhibivba marcatemente le formation de fibroblastos e stimulava le erythropoiese; e prednisolona inhibiva completemente le formation de fibroblastos e permitteva le continue differentiation de omne elementos sanguinee in reduite numeros.

**REFERENCES**

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