Mitotic Activity in Vitro of Erythroblasts Previously Exposed to Erythropoietin

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The homeostatic control of erythropoiesis by a humoral factor, erythropoietin, seems at present well established, and investigative work in progress in several laboratories is aimed at specific problems such as the chemical nature of erythropoietin, its site of production, metabolism and mechanism of action.

Relatively little experimental work has been done on the mechanism of action of erythropoietin. Theoretically, erythropoietin can increase erythropoiesis in one of three ways: it can induce more stem cells to differentiate into erythroblasts, increase the mitotic activity of differentiated erythroblasts, or do both. Evidence obtained recently with autoradiographic technics has been interpreted as favoring an increased flow of stem cells into the erythroblast compartment under erythropoietin stimulation, whereas the proliferation and maturation of erythroblasts are not affected.1,2 A different conclusion, implicating marrow erythroid cells as the target organ of erythropoietin, has been reached by one of us,3 who observed an increased mitotic activity of erythroblasts incubated in the presence of erythropoietin in marrow cultures.

In the present report, further experimental work in support of a direct effect of erythropoietin on the mitotic activity of erythroblasts will be presented and discussed in relation to findings by other workers and to current theories on the mechanism of action of erythropoietin.

Method

Bone marrow cell suspensions were cultured in homologous plasma clots. Colchicine was incorporated into the fluid medium, which consisted of 3 parts of homologous serum to be tested and 1 part Gey’s solution. Following incubation, imprint smears were made from the intact clots. The number of arrested erythroid mitoses was determined and expressed as a percentage of the total number of mitotable erythroid cells. All nucleated erythroid cells down to and excluding late normoblasts were considered capable of mitosis. The latter cell was characterized by a pyknotic nucleus and acidophilic cytoplasm.

The mitotic activity of marrow erythroblasts as a function of the time of incubation was studied under varying experimental conditions. Since the medium contained colchicine, mitoses arising during incubation were arrested at metaphase. As expected, arrested mitoses accumulated in the cultures with the time of incubation. The number of arrested mitoses counted at the end of a given period of incubation was an index of the number of cells which had entered mitosis during that period.
A total of 12 culture experiments was done. Bone marrow was cultured according to a method published previously,\textsuperscript{3,4} with some minor modifications:

Serum and bone marrow from anemic, normal and moderately polycythemic rabbits were used.

Anemia was induced by bleeding. Rabbits were bled 60–70 ml. daily, until a hemoglobin level of 4.5 Gm. per cent was reached. The animals were then exsanguinated.

Polycythemia was induced by a single massive drip transfusion of 150 ml. blood into the ear vein. In some cases a smaller second transfusion was necessary. Hemoglobin levels of 16–20 Gm. per cent and reticulocyte counts below 1 per cent were reached. The animals were used on the fourth or fifth day of their polycythemic state.

Bone marrow was obtained immediately after the animal (anemic, normal or polycythemic) had been killed by exsanguination. Femora and tibiae were removed, the marrow cavity exposed and the marrow spooned out into Gey’s solution. Tissue clumps were broken up by vigorous tapping. Suspensions were used within 4 hours and kept in the refrigerator until used.

Colcemid (Ciba) to a final concentration of 1:50,000 was added to the culture medium.

In some experiments, marrow suspensions were exposed to sera with varying concentrations of erythropoietin prior to incubation. This was done by gently shaking 0.5 ml. aliquots of bone marrow material for 4 hours at 37 C. in siliconized Erlenmeyer flasks containing 10 ml. of serum (anemic, normal or polycythemic). After centrifugation, the supernatant serum was removed and the marrow washed twice in Gey’s solution. Clumps were broken up by tapping. One-tenth ml. of red cells was added to each marrow sample. This was found to improve the quality of smears made subsequently. The marrow was then cultured in plasma clots in the usual manner.

RESULTS

1. Marrow Exposed to Erythropoietin in Vivo

Washed marrow from an anemic, a normal and a polycythemic rabbit were incubated simultaneously in a medium containing polycythemic serum. While in cultures of polycythemic marrow mitotic rates remained low, they rose rapidly in cultures of anemic marrow. At 9–11 hours, mitotic rates of anemic marrow were around 40–50 per cent, as against rates of 20–30 and 10–15 per cent respectively for normal and polycythemic marrow (fig. 1).

2. Marrow Exposed to Erythropoietin in Vitro

Mitotic rates obtained with marrow from a polycythemic animal, shaken for 4 hours with polycythemic, normal and anemic serum respectively, then washed and cultured in a clot with polycythemic serum, are shown in figure 2. Following exposure to anemic serum, mitotic rates were high. They reached a maximum of 30–40 per cent at 9–11 hours of culture with polycythemic serum. Rates were lower for marrow previously exposed to normal serum and lowest following exposure to polycythemic serum.

When marrow was shaken with polycythemic serum to which various concentrations of a purified erythropoietin preparation\textsuperscript{*} were added, the curves for mitotic rates were similar to those obtained with anemic serum. Increasing the erythropoietin from 2 to 6 units, or adding erythropoietin to anemic serum,

\textsuperscript{*}Sheep plasma erythropoietin concentrate AL-0336, prepared by Armour Pharmaceutical Co. in cooperation with the University of Chicago.
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WASHED MARROW FROM
- ANEMIC
- NORMAL
- POLYCYTHEMIC
RABBIT
CULTURED WITH POLYCYTHEMIC SERUM

Fig. 1.—Mitotic rates of erythroblasts from an anemic, a normal and a polycythemic rabbit incubated with polycythemic serum.

did not increase the mitotic rate (fig. 2). Mitotic rates of 40–50 per cent appear to be maximal under the conditions of our experiments.

DISCUSSION

Marked differences in mitotic activity between erythroblasts exposed to varying concentrations of erythropoietin have been reported briefly in a previous publication. These observations have now been confirmed and extended. In addition to exposure to erythropoietin in vivo in the anemic animal, erythroblasts were exposed to erythropoietin in vitro. Erythroblasts so exposed and subsequently grown in an erythropoietin poor medium showed increased mitotic activity.

Mitoses initiated in vivo would be expected to be completed or arrested by colchicine in the culture within the time limit of a mitotic duration, which is of the order of one hour. The steady and considerable increase in mitoses, which took place over a period of incubation extending far beyond this time limit, could, therefore, be accounted for only by mitoses actually arising in vitro. The higher mitotic rates achieved by anemic marrow were, accordingly, a reflection of a true increase in mitotic activity in vitro. This increase, in turn, was due to some stimulus present in vivo in the anemic animal and carried over to the culture. Since plasma was removed by washing, this stimulus, presumably erythropoietin, must have been carried over by the cells. It may, therefore, be assumed that erythropoietin was taken up by the erythroblasts from the blood in vivo and was still present intracellularly in the cultures. Alternatively, exposure to erythropoietin in vivo may have initiated in the erythroblasts a chain of events leading subsequently to cell division.
To provide further experimental support for the above conclusions, the exposure to various concentrations of erythropoietin, which is assumed to take place in vivo, was simulated in vitro by shaking aliquots of the same normal or polycythemic marrow in either erythropoietin rich or erythropoietin poor serum, then washing the marrow and culturing it in an erythropoietin poor medium. This procedure was also intended to meet any objections as to the validity of comparing polycythemic and anemic marrow. The two cell populations may differ in their age distribution due to the presence of more immature forms in the stimulated marrow of the anemic animal.

It is not surprising that in vitro methods have been applied by several workers to the problem of a direct effect of erythropoietin on erythroid cells. Alpen, Lajtha and Van Dyke, and Thomas, Lochte and Stohlman have failed to demonstrate an effect of erythropoietin in vitro on several parameters of erythroblast metabolism, including heme and DNA synthesis. However, for any such effect to be detectable, the system used should provide conditions favorable to proliferation. This is not the case with the Osgood and Brownlee-type fluid medium cultures used by these authors. Although some mitotic activity is claimed for this type of culture, it certainly lags far behind the high mitotic rates observed in our clot cultures. In addition, the time interval (4–6 hours) over which Thomas and co-workers studied DNA synthesis is too short to detect any effect of erythropoietin. Mitotic rates of both erythropoietin stimulated and control erythroblasts were found by us to be equally low during the first 5 hours of incubation. Appreciable differences in numbers of cells synthesizing DNA are, therefore, not likely to be noticeable in this short time interval.
Gordon and co-workers, on the other hand, observed significant increases in numbers of erythroid cells and erythroid mitoses in the marrow of the isolated hind limb of rats perfused for 4 hours with blood from repeatedly bled and phenylhydrazine treated animals. These observations support a direct effect of erythropoietin on marrow erythroblasts.

The demonstration of erythropoietin in washed erythroblasts is of special importance to our proposed scheme of its mechanism of action. Gordon has noted that only blood and urine have so far served as reliable sources of erythropoietin. Extracts prepared by Gordon and co-workers from a variety of organs, including bone marrow, administered to rats, did not show any erythropoietin effect. This negative result, however, does not necessarily contradict our finding of erythropoietin within marrow erythroblasts, since according to Gordon's procedure intraerythroblastic erythropoietin was subjected to considerable dilution and loss of activity. The amount of erythropoietin reaching individual erythroblasts in the recipient was therefore only a small fraction of that originally present in the donor cells.

The observed increase in mitotic rates of erythroblasts under erythropoietin stimulation could be accounted for by one of the following mechanisms. Proliferative activity may be distributed uniformly among individual cells at all levels of stimulation. In this case, each cell would undergo the same number of additional divisions under increased stimulation, and intermitotic intervals would be uniformly shortened for all cells. Alternatively, proliferative activity may not be equally shared by all cells, so that at lower levels of stimulation only a fraction of erythroblasts realize their full potential for mitotic division. Increased stimulation may, under these circumstances, increase the proportion of cells undergoing the maximum number of mitoses. At maximum mitotic activity, each cell is capable of undergoing four mitoses during its proliferative phase. However, under resting conditions only few cells actually do so. Abortive and incomplete erythropoiesis have been recognized, although there is no agreement as to their quantitative aspects. The importance of cell death as a factor in the regulation of erythropoiesis has been emphasized by Stohlman, while Suit and co-workers have observed cells reaching the circulation without an intervening mitosis or with only one or two mitoses. By stimulating erythroblasts to mitotic division, erythropoietin would mobilize these reserves, and, depending on the concentration of erythropoietin, proliferative activity would approach a total level.

A choice between these two mechanisms cannot be made on the basis of our data alone. Other considerations, to be outlined in the following paragraphs, make the second more probable.

The concept of a close association between maturation and proliferation still dominates the thinking of many workers in the field. According to this concept, each stage gives rise to the next by mitosis, and generation time is synonymous with the time spent in a stage of maturation. The abrupt transition

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*This figure is based on the time interval of 4 days from the appearance of proerythroblasts to the first late normoblasts, observed during repopulation of the erythroid marrow in a case of acute erythroblastopenia (to be published) and on an intermitotic cycle time of 20–24 hours for human erythroblasts."
from one stage to the next, inherent in this concept, is not borne out by experience with marrow cell morphology. What one actually observes in the marrow is gradual change in the morphologic characteristics of the cell. Recognized “classes,” or stages of maturation, are nothing but useful landmarks along the path of continuous change. When a cell has divided, the daughter cells emerging from telophase are at about the same level of maturation which the mother cell had reached when it entered prophase. Maturation would then go on in the daughter cells as if division had not taken place. Maturation and proliferation are, accordingly, parallel but independent processes. Maturation, once triggered off when a stem cell becomes an erythroblast, proceeds continuously. In contrast, DNA synthesis and cell division occur intermittently. The speculation may be permitted that events leading to cell division are under the control of erythropoietin.

Alpen and Cranmore, using Fe$^{59}$ and an autoradiographic technic, found that the time required to halve the median grain count in dog’s proerythroblasts did not differ materially in bled and unbled animals. They concluded that the generation time of dog’s proerythroblasts was not shortened under the stimulus of bleeding. Based on this and other evidence, models for the regulation of erythropoiesis have been proposed by Alpen and Cranmore and by Lajtha, which leave no room for play in the proliferative activity of erythroblasts under varying conditions of stimulation and shift the weight of regulation to the “stem cell” compartment.

Calculations of the proliferative activity of the erythroblast line would be materially affected by changes in the generation time of proerythroblasts only if it could be shown that the latter’s share in the total mitotic activity of the cell line was considerable.

Mitotic activity has variously been reported to increase, decrease or to be the same throughout progressive stages of maturation. Counts of the differential distribution of erythroid mitoses over the various stages of maturation are notoriously unreliable, since cytoplasmic as well as nuclear characteristics are lost during mitosis. In a case of acute erythroblastopenia, mentioned previously, during the initial phase of marrow repopulation, we have been able to determine the mitotic index in a pure proerythroblast population and found it to be low (about 2.5 per cent) compared with a mitotic index of 5 per cent determined on a mixed erythroid cell population, when maturation had reached the late normoblast stage. The main burden of any increase in erythroid proliferation would, therefore, be borne by the more mature cells, whereas the generation time of the less active cell, the proerythroblast, need not be appreciably shortened.

**Summary**

The proliferative activity of rabbit erythroblasts previously exposed to erythropoietin was studied in clot cultures containing colchicine.

Erythroblasts exposed to erythropoietin in vivo (in the anemic animal) or in vitro (by shaking with anemic serum) showed high mitotic rates when subsequently cultured in an erythropoietin poor medium.
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These findings indicate that erythropoietin is taken up by erythroblasts from serum. Intracellular erythropoietin stimulates erythroblasts to increased mitotic activity. The direct effect of erythropoietin on the ability of erythroblasts to proliferate constitutes a mechanism by which erythropoietin regulates erythropoiesis.

SUMMARIO IN INTERLINGUA

Le activitate proliferative de erythroblastos de conilio, previemente exponite a erythropoietina, esseva studiate in culturas a coagulo continente colchicina. Erythroblastos exponite a erythropoietina in vivo (in le animal anemic) o in vitro (per agitation con sero anemic) monstrava subsequentemente un Intense activitate mitotic quando illos esseva culturate in un medio povre in erythropoietina.

Iste constatationes indica que erythropoietina es acceptate per le erythroblastos ab le sero. Erythropoietina intracellular stimula le erythroblastos a un accelerar activitate mitotic. Le directe effecto de erythropoietina super le capacitate proliferatori de erythroblastos constitue un mechanismo per le qual erythropoietina regula le erythropoiese.

REFERENCES


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