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## Experimental Research on the Proliferative and Differentiative Activity of the Erythroblast in Chronic Erythremia, the Chronic Form of Di Guglielmo's Disease

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**I**N RECENT YEARS with the use of in vitro culture of bone marrow in both solid and liquid media, it has been possible to obtain more detailed knowledge of the basic biologic activities of hemopoietic cells. It has become possible to study the proliferative activity of marrow cells as well as cellular differentiation (maturation), in partly dissociated fashion. For studies of proliferative activity the stathmokinetic test<sup>1</sup> applied to plasma clot cultures have made it possible to determine the proliferative capacities of the various marrow cells with far greater precision than was formerly possible with the use of solely morphologic study of the bone marrow. For investigation of differentiative activities, the in vitro methods based on incubation of marrow cells in fluid media have been effective. In large part, the credit for the application of these methods to study of bone marrow belongs to the Italian school of hematology. The stathmokinetic test is based on the property exhibited by colchicine of blocking the evolution of karyokinesis in the pre-metaphase stage, the consequence being that, when an optimum dose of this alkaloid is added in the bone marrow culture medium, it brings all medullary mitoses to a stop immediately after their onset and causes the accumulation of all such karyokineses as may occur during the experiment. As any mitosis is stopped by colchicine promptly after the prophase stage, the progressive increase of karyokinesis through the time is a function of the duration of the interkinetic period of the blood-forming cells, without any interference whatever by the "duration of kinesis" factor which is instead known to affect the value of the mitotic index.

An assessment of the amount of proliferation is therefore provided by the rate of increase in the number of mitoses and an evaluation of the differences existing among the various cell types under the various conditions of hematologic physiopathology makes it possible to establish the characters of the proliferating activity of the cells under examination.

Of course, the in vitro methods we have used in making this study display phenomena which may not be identical with those occurring in vivo because of the different environmental conditions of the cells. Nevertheless, they are useful since they permit comparative studies which can be expressed in quantitative

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terms. The stathmokinetic test has established the fact that under normal conditions in adults the basophilic erythroblast is the cell with the most marked proliferative capacity as indicated by a stathmokinetic index of 200 per thousand,<sup>1</sup> whereas the polychromatic erythroblast has an index of only 90 per thousand. The myeloblast is the granuloblastic cell with the highest proliferative capacity, having an index of 150 per thousand,<sup>2</sup> and the stathmokinetic indices become appreciably lower for the more differentiated cells, promyelocytes and myelocytes having values of 60 and 30 per thousand, respectively. In embryonic hemopoiesis<sup>3</sup> the erythroblastic cells of the first generation, the megaloblasts, have high proliferative capacities, yielding a maximum stathmokinetic index of 509 per thousand in the basophilic stage and 110 per thousand in the polychromic stage. The second embryonic generation of erythroblasts, the normoblasts, have values of 380 per thousand for the basophilic and 50 per thousand for the polychromatic stages.

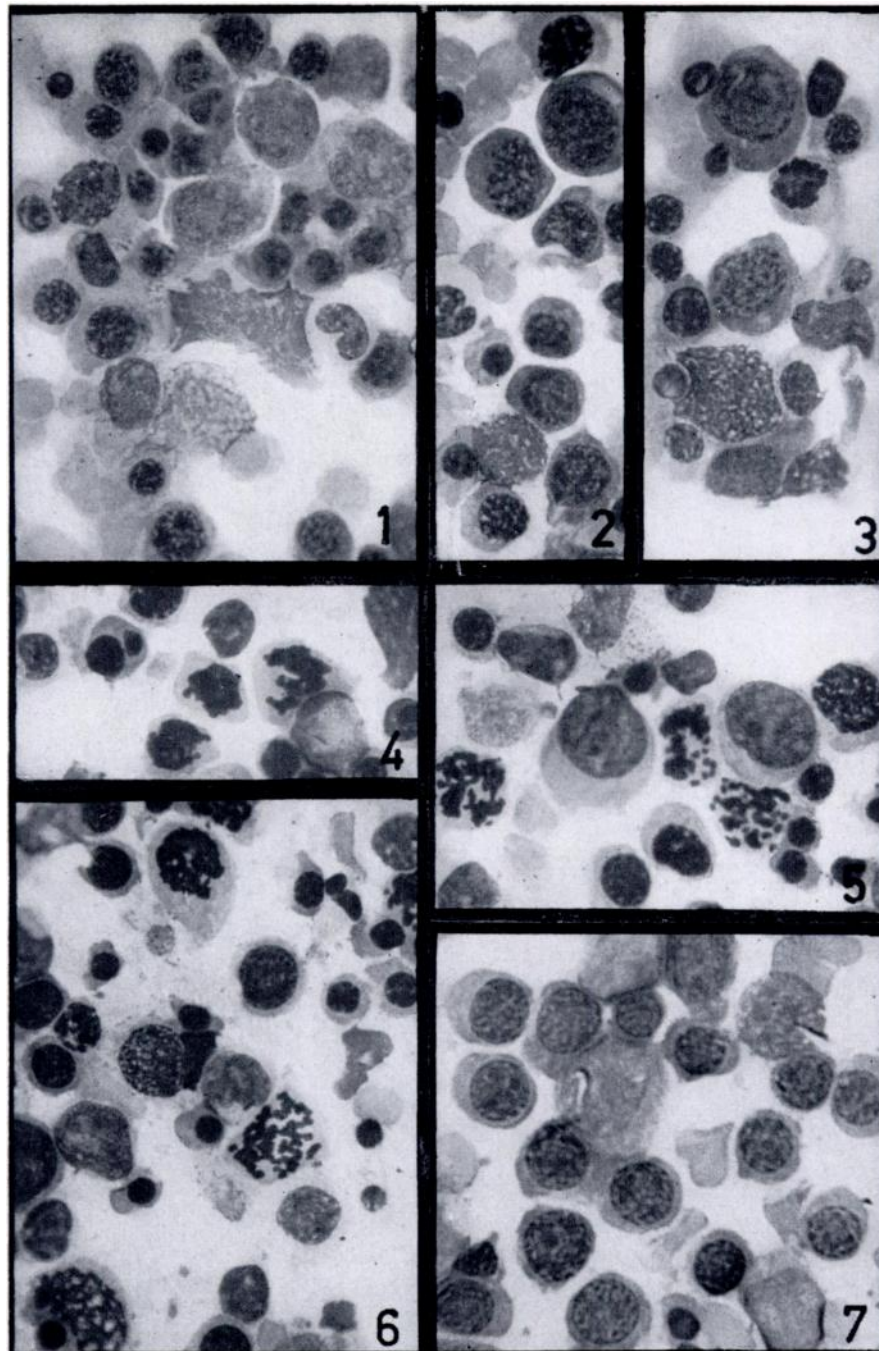
In addition there are numerous similar studies of the proliferative activities in pathologic hemopoiesis, contributed by investigators of the Italian school.<sup>4-9</sup>

The particular environmental conditions in liquid cultures (marrow or peripheral blood cells in heparinized plasma and Tyrode's solution) can be used to advantage for evaluation of the differentiating or maturative activities of hemopoietic cells. In this type of culture the proliferative capacity of cells is quite attenuated and the direct participation of cells of the reticulohistioid system is eliminated. Consequently the quantitative and qualitative modifications in such cultures are related predominantly to differentiative rather than proliferative processes.

In fluid cultures it has been shown that basophilic erythroblasts from normal marrow disappear after 18 to 24 hours of incubation; the polychromatic erythroblasts disappear after 48 to 60 hours, and the orthochromic forms after 96 to 100 hours. Hence, the maximum duration of the various stages of maturation *in vitro* are 18 to 24 hours for the basophilic, 24 to 36 hours for the polychromatic and orthochromic erythroblasts.<sup>10</sup> A number of studies of maturation of erythroblasts from patients with various hemopathies have been published.<sup>5-7</sup>

The purpose of this communication is to report our studies of the erythroblastic tissue of a patient with chronic erythremic myelosis,<sup>11, 12</sup> a primary hemopathy characterized by a proliferation involving particularly the erythropoietic system of the myeloid tissue in a manner analogous to the involvement of the granulopoietic system in leukemic myelosis. The disease appears to be a progressive, nonutilitarian proliferative hyperplasia of hemopoietic tissue, unlike a reparative or regenerative process. However, it must be admitted that neither these attributes nor the morphologic features of the cells make it possible, at all times, to distinguish between normal hyperplasia of unknown etiologies and dysplastic or neoplastic hyperplasia. Therefore it seemed probable that if our methods would give information about the mechanism by which the pathologic growth is attained, or of how the quantitative increases of medullary cells occur, a better understanding of the disease would result.

Theoretically, quantitative increases of medullary elements can be determined by observations of various distinct processes, such as cellular proliferation and differentiation, migratory activity of the hemopoietic cell from marrow into blood and, finally, by observations of the direct derivation of medullary cells

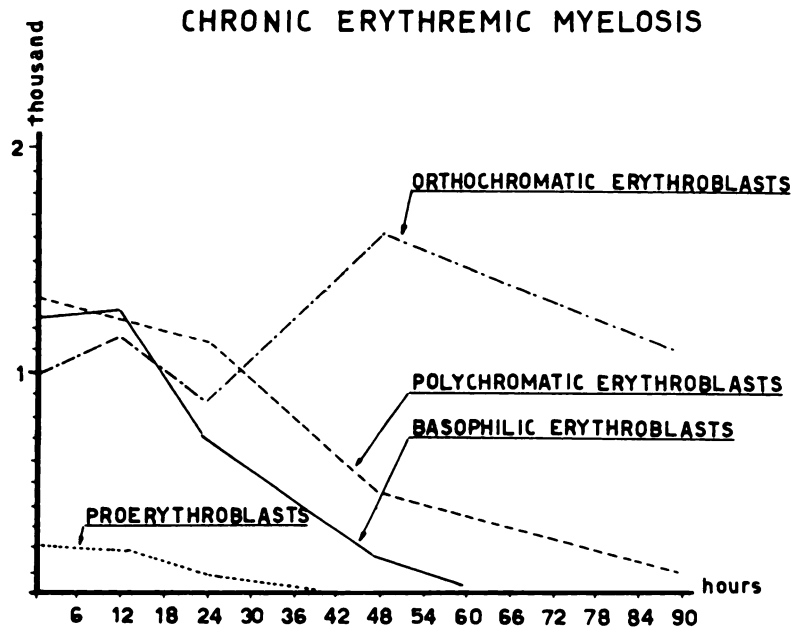


## CHRONIC ERYTHREMIC MYELOSIS

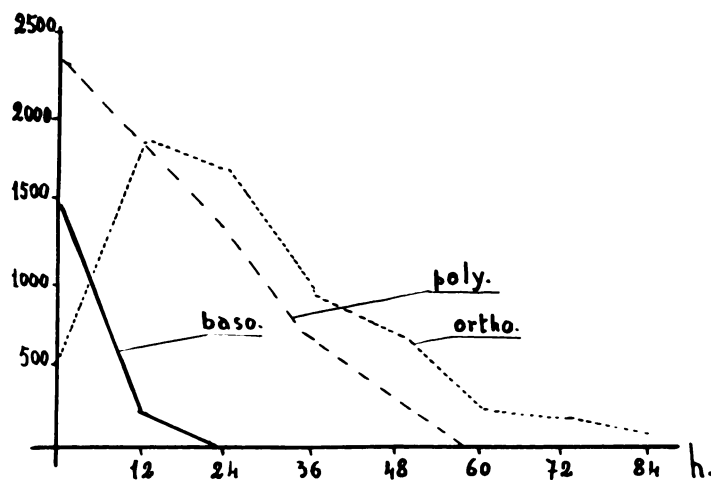
FIGS. 1, 2, 3—Microscopic fields of bone marrow before culture: they show the presence of numerous histoid proerythroblasts and of erythroblasts in various maturative phases. FIGS. 4, 5, 6—From a solid culture with colchicine added: numerous erythroblastic mitoses in pre-metaphase after 6, 12, 16 hours respectively of survival in vitro. FIG. 7—From a liquid culture: diminished the quota of proerythroblasts and basophilic erythroblasts at the 24th hour of survival in vitro.

from the reticuloendothelial system. It is easy to understand how deviations from the normal degrees of activity of these processes might lead to an increase of any particular types of hemopoietic cells, but the nature and mechanisms of such increases would have to be learned from functional studies. With these

### INVESTIGATION OF THE DIFFERENTIATIVE ACTIVITY



GRAPH 1.—A study of the differentiative activity of the erythroblasts of chronic erythremic myelosis using the "in vitro" method culture establishes a slowing down of the erythroblastic differentiative activity with respect to the norm.



GRAPH 2.—Culture of normal human bone marrow: differentiation of the erythroblasts.

thoughts in mind we set out to study the biologic activity of the erythroblast in chronic erythremic myelosis.

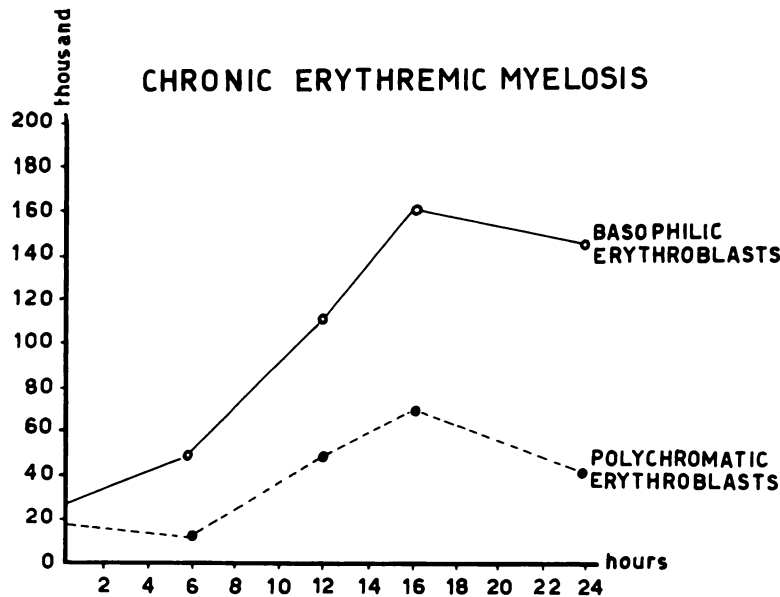
#### METHODS AND RESULTS

The method used for study of the differentiative activity of the hemopoietic cells is the one suggested by Gunz<sup>13</sup> and later modified in part by Astaldi and Tolentino.<sup>10</sup> It consists of keeping the bone marrow cells in a liquid medium (heparinized plasma plus Tyrode's solution) at a temperature of 37 C. Every 6 to 12 hours quantitative determinations are made of the numbers of cells in different stages of maturation, i.e., the numbers of basophilic, polychromatic and orthochromic erythroblasts are determined.

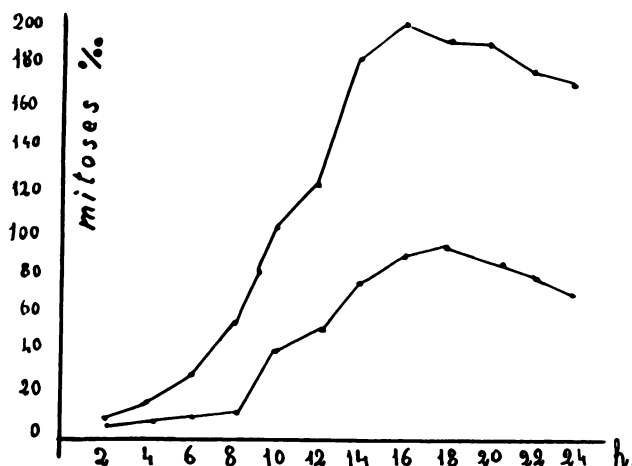
The marrow from a patient with chronic erythremic myelosis (figs. 1, 2, 3) was placed in heparinized plasma of the patient and Tyrode's solution. The absolute numbers of the erythroblasts in their different stages of maturation were determined at the beginning of the experiment and periodically every 12 hours, as shown in graph 1. The graph shows that the nucleolated basophilic erythroblasts (pronormoblasts) disappear between 24 and 36 hours, and the non-nucleolated basophilic cells (basophilic normoblasts) between 48 and 60 hours (fig. 7). Therefore, the maximum life of the nucleolated basophilic erythroblasts should be about 24 to 36 hours with an equal length of time for the non-nucleolated basophilic erythroblasts. Consequently the maximum life of the whole basophilic erythroblast population should be about 60 hours, under the conditions of the experiment. Similar observations on normal marrow are shown in graph 2.

The curve of the polychromatic erythroblasts shows a very slow decline as compared with normal; after 48 hours the level of these cells is reduced to slightly less than half the initial level, whereas in normal marrow cultures it is considerably lower. It was not possible to evaluate the maximum duration of the polychromatic stage because after 90 hours degenerative changes had begun to occur. The orthochromic erythroblasts remained at a

#### INVESTIGATION OF THE PROLIFERATIVE ACTIVITY USING THE STATMOKINETIC TEST



GRAPH 3.—Study of the proliferative activity of normal human bone marrow by the stathmokinetic test.



GRAPH 4.—This graph shows the curves of the stathmokinetic indices of the basophilic and polychromatic erythroblasts in chronic erythremic myelosis. Results prove that proliferative activity is within normal limits.

nearly constant level for the first 24 hours of culture; then they gradually increased in number. The highest counts occurred between the 48th and 60th hours. This is considerably delayed as compared with cultures of normal marrow in which the peak incidence of orthochromic forms occurs at the 18th hour. Later the values for orthochromic cells in the cultures of marrow from chronic erythremic myelosis fall, but it was not possible to calculate the maximum duration of these cells because of degenerative changes in the cultures after 90 hours.

The preceding observations which pertain to the differentiative capacity of erythroblasts were made from cultures maintained in a fluid medium. For studies of the proliferative activity of the cells we used cultures in a solid medium. Fragments of marrow obtained by sternal puncture were washed in Tyrode's solution and placed in plasma of the patient which was allowed to clot after the addition of chick embryo extract. Colchicine was added in a final concentration of 1:500,000. Every two hours, starting at the 6th hour until the 24th hour, smears were prepared on slides from the medullary fragments. These were stained with May-Grünwald-Giemsa solution (figs. 4, 5, 6). From these smears we determined the numbers of cells in mitosis among basophilic and polychromatic cells separately, and the values are shown in graph 3.

At the beginning of the experiment the number of mitoses before the addition of colchicine was 21 per thousand for the basophilic erythroblasts, and 18 per thousand for the polychromatic erythroblasts. The incidences of mitoses increased and reached their highest levels at the 16th hour, so that the stathmokinetic index for basophilic cells reached 160 per thousand, and for the polychromatic cells it reached 68 per thousand. After the 16th hour the indices decreased. For similar observations on normal marrow see graph 4.

#### DISCUSSION

The results of the experiment allow us to reach the following conclusions:

1. *The erythroblastic cells of chronic erythremic myelosis reveal indisputable evidence of differentiative activity in vitro.* This fact conforms to what has been observed by others (Gunz,<sup>13</sup> Israel,<sup>14</sup> Astaldi,<sup>15</sup> Blackburn and Lajtha<sup>16</sup>) in cultures of chronic myeloid and lymphatic leukemia, even though their data cannot always be interpreted with certainty because their technics did not permit the collection of enough data.

It should be pointed out that culture of granuloblastic tissue in solid media is

of little use for the study of differentiative activity of granuloblastic cells because complete pictures of cellular maturation are not obtained. In our experiment, on the other hand, we were dealing with tissue having a more rapid differentiative activity than that of granuloblasts. Therefore it was possible to obtain total transformations and more reliable data. In fact, we witnessed the complete disappearance of nucleolated basophilic erythroblasts, the maximum incidence of polychromatic erythroblasts, and increase of orthochromic cells to values higher than those at the beginning of the experiments. All these changes indicate that differentiation had taken place in the cultures.

2. *The rate of maturation of erythremic erythroblasts is slower than normal.* After remaining constant for the first 12 hours of culture the basophilic erythroblasts decrease slowly to disappear at the 60th hour. The polychromatic erythroblasts diminish slowly; at the end of 96 hours they are still present. The orthochromic cells remain constant for 24 hours; then their incidence rises to reach a maximum value near the 48th hour, and at the end of the experiment they are still numerous. Compared to time intervals observed with normal marrow cultures these are considerably prolonged. This slowing down of the differentiation process may be only apparent, however. There is a possibility that for a certain period the hemohistiopoietic output of the marrow may be maintained in culture, so that the accumulation of primitive cells resulting from this activity may mask the appearance of differentiative activity *in vitro*.

Moreover the high percentage of nucleolated basophilic elements present at the beginning of the culture period also can be responsible for the apparent slowed down disappearance of the basophilic erythroblasts. However, proliferative activity in our experiment was slow. Hence these theoretical situations cannot justify the delayed disappearance of the various types of erythroblasts found in our experiments.

The delayed disappearances of the polychromatic and orthochromatic cells compared with that of the basophilic cells, and the failure of disappearance of the polychromatic and orthochromic cells within expected normal time limits establishes the concept of a slowing down of maturation of erythroblasts in our experiments.

3. *The proliferative activity of erythroblastic tissue of chronic erythremic myelosis is normal.* The initially level curve for basophilic erythroblasts rises sharply after the 4th hour. The stathmokinetic index reaches a maximum of 160 per thousand at the 16th hour, after which it falls. The index for polychromatic cells begins at a low level, rises only after the 6th hour at a rate much lower than that of the basophilic cells and at the 16th hour also reaches a maximum value, in this case of 70 per thousand. This is, therefore, an indication of normal activity and, considering the initially high percentage of nucleolated basophilic erythroblasts, it could be considered an indication of subnormal proliferative activity.

4. *Of the possible pathogenic factors for the erythroblastic hyperplasia of chronic erythremic myelosis considered at the beginning of this discussion, the one connected with a maturation defect appears the most evident.* Broadly speaking, the biologic characteristics of the erythroblasts in chronic erythremic myelosis permit us to distinguish that disease from the medullary hyperplasia of thalassemia

and some anemias in which the principal pathogenic factor is increased proliferative activity of the erythroblasts.<sup>5, 6, 17</sup> The absence of proliferative hyperactivity makes it possible to compare bio-morphologically the erythroblast of the erythremic disease and that of polycythemia vera, as it has been shown that in polycythemia vera the proliferative activity of erythroblasts is also normal.<sup>7</sup>

#### SUMMARY

Using the method of culture in vitro, the authors have studied the proliferative and differentiative activities of the erythroblastic tissue in chronic erythremic myelosis.

The results of the research allowed the authors to reach the following conclusions:

1. the erythroblastic cells of chronic erythremic myelosis reveal indisputable differentiative activity in vitro;
2. the rate of maturation of the erythremic erythroblasts is slower than normal;
3. the proliferation of the erythroblastic tissue in chronic erythremic myelosis is normal;
4. in the genesis of the erythroblastic hyperplasia in chronic erythremic myelosis, the most evident factor is a defect of maturation of the erythroblasts.

#### SUMMARIO IN INTERLINGUA

Utilisante le methodo del cultura in vitro, le autores ha studiate le activitates proliferative e differentiative del histos erythroblastic in chronic myelosis erythremic.

Le resultatatos del recerca permitteva le formulation del sequente conclusiones:

1. Le cellulas erythroblastic in chronic myelosis erythremic revela un indisputabile activitate differentiative in vitro.
2. Le maturation del erythroblastos erythremic progredie plus lentemente que lo que es normal.
3. Le proliferation del histos erythroblastic in chronic myelosis erythremic es normal.
4. In le genese de hyperplasia erythroblastic in chronic myelosis erythremic, le plus evidente factor es un defecto de maturation del erythroblastos.

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